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Calcium Antagonism and Calcium Entry Blockade*

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I. Introduction and Historical Perspective

A. Introduction

Calcium is involved in several vital cellular processes including contractile, secretory, and neural activities. In most of them, calcium may be considered as a final intracellular messenger. As a free cation, or more often after formation of a complex with some macromolecule such as calmodulin, calcium is an activator of several key enzymes of the cell. In addition, calcium is an essential constitutent of bone matrix. Since the end of the 1960s, it has been recognized from experimental studies initiated in the laboratories of Fleckenstein and Godfraind that the function of calcium in excitation-contraction coupling could be altered by pharmacological agents that are without effect on the concentration of calcium in extracellular fluids and calcified tissues. The first drugs shown to interfere with calcium function (e.g., cinnarizine and verapamil) were already used in the 1960s for the treatment of angina pectoris, allergic reactions, and vertigo. Several chemical families have been added to the first generation of compounds characterized on a purely phenomenological basis as "calcium antagonists" (table 1; fig. 1). The present purpose is to review the main pharmacological properties of these drugs and their likely mechanisms of action (table 1). They have been generally classified under several titles since the term calcium antagonist first came into general use to characterize a drug action on contraction subsequent to calcium entry into smooth muscle cells. A *calcium antagonist*, a compound that does not necessarily (and in fact is unlikely to) compete with Ca^{2+} for a binding site, may now be defined as a drug that alters the cellular function of calcium by inhibiting its entry and (or) its release and (or) by interfering with one of its intracellular actions. Subgroups of calcium antagonists can therefore be defined. Those that specifically inhibit Ca^{2+} entry into cells due to tissue excitation by various stimuli have been called *calcium entry blockers* (399). This antagonistic activity is most likely due to interaction with calcium channels activated by membrane depolarization or by receptor stimulation, and, in these circumstances, these agents may also be termed *calcium channel blockers or inhibitors*. When blockade occurs at the level of the "slow" channels in cardiac tissues, the term *slow channel blockers* has been used (verapamil, nifedipine, diltiazem, and some of their derivatives). The term *calcium overload inhibitors* has also been utilized in some classifications, but has not gained wide currency. Agents that interact specifically with calmodulin are properly labelled *calmodulin antagonists*. The designation "*calcium agonist*" has been introduced recently to characterize dihydropyridine derivatives that increase the probability of calcium channel opening instead of blocking them. The whole group of agents affecting calcium movements has received the general denomination of *calcium modulators*.

In this review, we intend to examine the great variety of effects and apparent selectivity of drugs initially termed "calcium antagonists" to which a single mechanism of action can be attributed, this is, calcium entry blockade. To this end, we will examine the actions of these compounds in experimental situations, mainly isolated tissues, designed to elucidate their mechanism of action.

B. First Steps in the Identification of Calcium Antagonists

Rubin (935) has recently summarized the historical evolution of the concepts regarding the role of calcium in biology. Although its importance for the maintenance of cellular activity was observed by Ringer as early as 1883, its key role in intracellular functions was not perceived until 70 yr later by Heilbrunn in the United States and by Kamada in Japan (495, 596). It is therefore not surprising that investigations into the pharmacology of the cellular metabolism of calcium have only begun in the last 20 to 25 yr. During the same period, great advances have been made in the understanding of the regulation of muscle contraction by calcium, and this has recently been reviewed (233). Two recent books are devoted to the role of calcium in biological processes (718, 935).

On examination of the literature of the end of the 1950s and of the early 1960s, it appears that the role of calcium in pharmacological processes was initially quantified in smooth muscle studies (232, 257). Schild and coworkers (236, 237), for example, showed that depolarization of rat uterus rendered the membrane of the smooth muscle cell permeable to extracellular calcium and that the force of the contraction developed by the preparation was proportional to the concentration of calcium in the extracellular fluid. In addition, they provided evidence that acetylcholine-evoked contractions of smooth muscles were dependent on both intracellular and extracellular calcium. Similar observations were made with serotonin, histamine, and oxytocin (1184).

In the 1960s, inhibition of Ca^{2+} entry as a mechanism of drug action was demonstrated in simultaneous and independent studies on cardiac tissues by Fleckenstein et al. (294–297, 307, 308, 323, 329) and on arterial tissue by Godfraind and coworkers (395, 415, 416, 419, 420, 435). These latter authors analyzed the pharmacological

TABLE 1
*Calcium modulators**

Agents affecting Ca^{2+} movements (calcium antagonists and calcium agonists)
A. Inhibitors: calcium antagonists
1. Agents acting at the plasma membrane
1.A. Calcium entry blockers
Group 1: selective calcium entry blockers
Subgroup I A: agents selective for slow calcium channels in myocardium (slow channel blockers)
Phenylalkylamines: verapamil, gallopamil (D 600†); under investigation: anipamil, desmethoxyverapamil (D 888), emopamil, falipamil (AQ-A-39), ronipamil
Dihydropyridines: nifedipine, nicardipine, niludipine, nimodipine, nisoldipine, nitrendipine, ryosidine; under investigation: amlodipine, azodipine, dazodipine (PY 108-068), felodipine, flordipine, FR 7534, FR 34235, iodipine, isrodipine, mesudipine, ni(*l*)vadipine, oxodipine, PN 200-110, riodipine
Benzothiazepines: diltiazem
Subgroup I B: agents with no perceived actions on the slow calcium inward current in myocardium (voltage clamp)
Diphenylpiperazines: cinnarizine and flunarizine
Group II: nonselective calcium entry blockers
Subgroup II A: agents acting at similar concentrations on calcium channels and fast sodium channels
Bencyclane, bepridil, caroverine, etafenone, fendiline, lidoflazine, perhexiline, prenylamine, proadifen (SKF 525A), terodiline, tiapamil
Subgroup II B: agents interacting with calcium channels while having another primary site of action
They include, among others: agents acting on sodium channels (local anesthetics, phenytoin); on catecholamine receptors (benextramine, nicergoline, phenoxybenzamine, phenothiazines, pimozide, propranolol, WB 4101, yohimbine derivatives); on benzodiazepine receptors (diazepam, flurazepam); on opiate receptors (loperamide, fluperamide); on cyclic nucleotide phosphodiesterases (amrinone, cromoglycate, papaverine); barbiturates; cyproheptadine; indomethacin; reserpine
1.B. Sodium-calcium exchange inhibitors
Amiloride and derivatives
2. Agents acting within the cell
2.A. Acting on sarcoplasmic reticulum
Dantrolene, TMB-8
2.B. Acting on mitochondria
Ruthenium red
2.C. Calmodulin antagonists
Phenothiazines: trifluoperazine, chlorpromazine
Naphtalene derivatives: W-7
Local anesthetics: dibucaine
Dopamine antagonists: pimozide, haloperidol
Calmidazolium (R-24571)
B. Facilitators
1. Agents acting at the plasma membrane
Calcium agonists
Dihydropyridines: Bay K 8644; CGP 28392; YC-170
2. Agents acting on sarcoplasmic reticulum
Inositol 1,4,5-trisphosphate
Caffeine
3. Ionophores
A 23187, ionomycin

* For explanation, see section IV B.

† For a listing of compound designations and corresponding chemical names, see table 18.

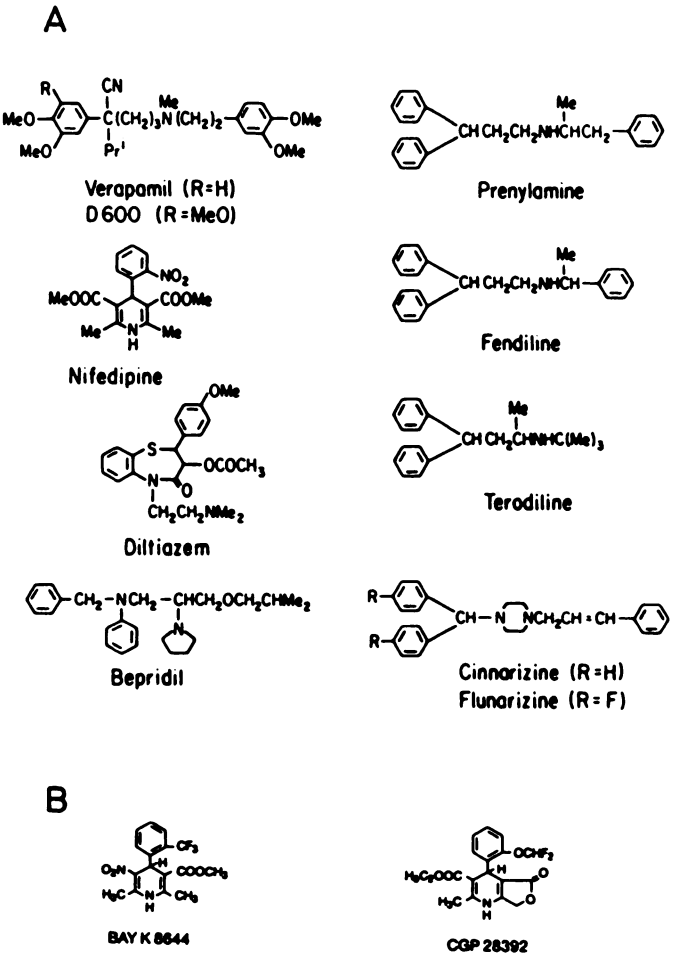

FIG. 1. Structure of some calcium entry blockers (*A*) and calcium agonists (*B*). *Me*, methyl; *Pr*^{i}, isopropyl.

properties of diphenylpiperazine derivatives, mainly lidoflazine [used as an antianginal drug (563)] and cinnarizine which was at that time considered an antihistaminic (1109, 1110) and was used as an anti-motion-sickness and antiallergic drug. Lidoflazine and cinnarizine inhibit contraction of some smooth muscles evoked by several agonists, such as norepinephrine, angiotensin, acetylcholine, and vasopressin (144, 395, 419, 1103). Because the active concentrations of the inhibitors were similar when tested against various agonists, it was proposed that lidoflazine and cinnarizine could interfere with a common mechanism activated by the various spasmogens (395, 419, 420). A hypothesis was that calcium translocation was this common mechanism, an idea indirectly supported by a large body of experimental work (78, 189, 235–237, 521, 540). According to the indirect evidence available at that time, the calcium activating the contractile machinery subsequent to agonist stimulation could have been translocated either from the outside to the inside of the cell, or within the cell from an intracellular store. To determine if the antagonism was related to one or the other of those possible mechanisms, the action of diphenylpiperazines on agonist-evoked con-

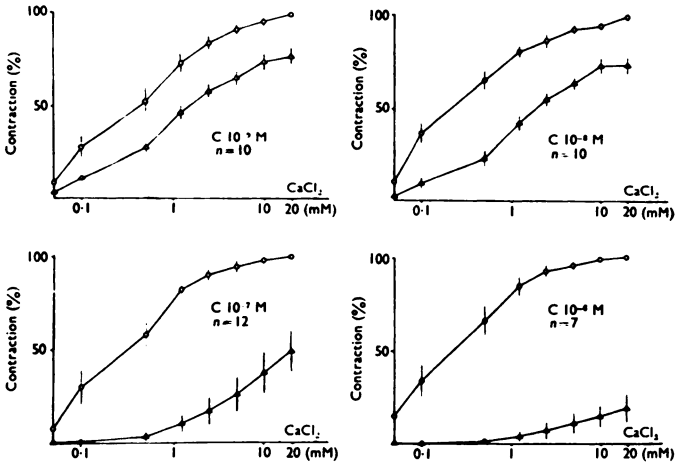

FIG. 2. Effect of cinnarizine on contractions evoked by Ca^{2+} in K^+-depolarized rabbit mesenteric arteries. Arterial preparations were preincubated in Ca^{2+}-free physiological solution, depolarized in Ca^{2+}-free KCl-rich solution, and then further incubated with increasing Ca^{2+} concentrations. Cumulative concentration-effect curves were obtained before (O) and after (Δ) addition of cinnarizine (*C*) at the concentrations indicated. Responses are expressed in percentage of maximal contraction evoked before addition of cinnarizine. The inhibitory effect of cinnarizine is observed at concentrations as low as 1 nM and resembles the action of antagonists in receptor studies. In view of this similarity, the term "calcium antagonist" was suggested to describe the action of cinnarizine. Reproduced from Godfraind and Kaba (417).

traction of arterial smooth muscle in the presence and absence of calcium was examined. Only the contractions dependent on extracellular calcium were found to be blocked by these drugs. Contractions evoked by KCl depolarization (due to calcium translocation from outside to inside the cell) were very sensitive to diphenylpiperazines. Godfraind and Polster (435) showed that the inhibitory effect of cinnarizine, lidoflazine, and chlorpromazine on the contractions of several arteries, including pig coronary arteries and human pulmonary arteries, evoked by KCl-rich solutions, could be antagonized by increasing the concentration of calcium in the perfusate (fig. 2). To characterize the diphenylpiperazines, they used the term "antagoniste du calcium" (calcium antagonist), a term used coincidentally by Fleckenstein to describe the effects of verapamil and prenylamine on cardiac tissue.

Verapamil was described in 1962 by Haas and Hartfelder (473) and classified as a coronary vasodilator, capable of improving the ratio of myocardial oxygen supply to oxygen consumption in anesthetized open chest dogs. Due to its absence of bronchoconstrictive effect (472), it presented a great advantage over propranolol and therefore was of great clinical interest. Verapamil was considered in 1968 by Nayler et al. (816) as interacting with beta-adrenoceptors, but by a mechanism different from *dl*-propranolol. In the 1960s, Fleckenstein and coworkers (294–297, 306–308, 323) were interested in the study of the utilization of high energy phosphates in relation to muscle contraction and oxygen consumption and of disturbances in high energy phosphate metabolism and the action of catecholamines. In this respect they studied the role of Ca^{2+} in the solution perfusing striated and cardiac muscle. In the course of his studies (294–334), Fleckenstein discovered that the action of verapamil and of prenylamine mimicked the effect of calcium withdrawal. Because of this, he proposed that excitation-contraction uncoupling evoked by prenylamine, verapamil, and its derivative, gallopamil (D 600)† was due to calcium antagonism. In 1972, Fleckenstein and Grün (316) showed that the vasodilating properties of verapamil were related to calcium antagonism. This concept of calcium antagonism was later extended to other drugs including dihydropyridines, diltiazem, and flunarizine (299, 328, 400, 406, 645, 646, 1042, 1101, 1115).

II. Pharmacodynamic Actions of Calcium Entry Blockers

A. Vascular Smooth Muscles

Smooth muscles show great variety in their intrinsic physiological and pharmacological properties. This is true not only between the various types of muscles originating from different physiological systems but also between the various smooth muscles integrated in one given system. For instance, in the cardiovascular system, the pharmacological properties of veins and arteries are different. Within arteries, large conducting vessels and small resistance arteries are also pharmacologically different. An exhaustive survey of the vast literature on the actions of calcium entry blockers in vascular tissues has not been undertaken. Rather, similarities and differences between types of vessels have been illustrated below by examining the actions of calcium entry blockers in representative large arteries, the aortae of rabbit and rat, in coronary arteries, cerebral arteries, resistance vessels, and in veins. The rat and rabbit aortae have been much used to elucidate the mechanism(s) of action of these compounds in both contractile experiments and by measuring ^{45}Ca uptake and release (430). The other vessels are of special interest, since the major clinical uses of calcium entry blockers are in the domain of hypertension and vascular insufficiency, particularly cardiac and cerebral. The actions of calcium entry blockers in venous tissues present some anomalies in comparison with arterial (and other) smooth muscle, which may be related to physiological differences (981), and some of these differences are detailed in this section.

1. Rat aorta, as a model. We intend to summarize here the main bulk of research performed on the rat aorta with the aim of analyzing the varous processes involved in the regulation of calcium metabolism at the cellular level and the sensitivity of these processes to pharmacological agents, mainly calcium antagonists. Rat aorta is a very interesting tissue to use as a model of vascular smooth muscle because it has properties intermediate

† For a listing of compound designations and corresponding chemical names, see table 18.

between conducting the resistance vessels. Due to the genetic control of rat breeding, standardized preparations may be obtained from both normal and pathological animals, and therefore reproducible results may be expected.

a. CALCIUM HOMEOSTASIS IN SMOOTH MUSCLE. The contractile activity of smooth muscle is regulated by the free Ca^{2+} concentration in the cytosol. In the resting state, the Ca^{2+} concentration is probably not higher than 100 nM, and the ratio of extracellular to intracellular Ca^{2+} exceeds 10,000. Upon stimulation, the cytosolic Ca^{2+} concentration rises, partly as a result of an influx of extracellular Ca^{2+}, and partly following the mobilization of internal Ca^{2+} stores, to reach about 10 μM. It has been proposed that this mobilization in vascular smooth muscle is mediated by a Ca^{2+}-induced Ca^{2+} release mechanism (943). Alternatively, agonist-stimulated Ca^{2+} release from the sarcoplasmic reticulum may be triggered by inositol trisphosphate, produced by hydrolysis of phosphatidyl inositol 4,5-bisphosphate (67, 1005). To restore the low resting cytosolic Ca^{2+} and to maintain Ca^{2+} homeostasis, the cell uses Ca^{2+} transport systems that are able to operate against large electrochemical gradients. Such Ca^{2+} transport systems have been demonstrated in the plasma membrane and in intracellular organelles, mainly the endoplasmic (sarcoplasmic) reticulum.

There are two main mechanisms allowing "uphill" Ca^{2+} transport. The first relies on the direct utilization of energy from ATP (Ca^{2+} transport ATPase), and the second uses the Na^+ electrochemical potential to drive the extrusion of Ca^{2+} in exchange for Na^+ entry (Na^+-Ca^{2+} exchange). ATP-dependent Ca^{2+} pumps with different properties are present in the plasma membrane and the endoplasmic reticulum. Their identification in smooth muscle has relied mainly on cell fractionation techniques, including the digitonin shift method (438, 773, 1156, 1158, 1172) (see also ref. 190). The plasma membrane Ca^{2+} pump is activated by calmodulin, which, in rat aorta, acts mainly by enhancing the affinity of the pump for Ca^{2+} (773). The nucleotides cyclic AMP and cyclic GMP may also regulate Ca^{2+} availability in smooth muscle (688, 872) perhaps by regulation of Ca^{2+} pumps. Cyclic AMP-dependent phosphorylation seems to enhance Ca^{2+} accumulation into the endoplasmic reticulum, as indicated by studies on skinned arterial smooth muscle (946). The role of cyclic GMP in relaxation of vascular smooth muscle appears to be well-established in the action of nitro-compounds and in endothelium-dependent relaxation by acetylcholine (536, 893). Stimulation of the plasma membrane Ca^{2+} pump following cyclic GMP-dependent phosphorylation may contribute to vascular relaxation (1033).

Participation of a plasmalemmal Na^+-Ca^{2+} exchanger in the physiological control of Ca^{2+} in smooth muscle is controversial (100, 523, 1091). Nevertheless, such a system has been clearly demonstrated in various smooth muscle tissues (467, 771, 772). However, in smooth muscle, the Ca^{2+} transport capacity of the Na^+-Ca^{2+} exchange system and its Ca^{2+} affinity seem to be distinctly lower than those of the plasmalemmal Ca^{2+} transport ATPase (771, 772), in contrast to the heart sarcolemma where the transport capacity of the exchanger is much higher than that of the Ca^{2+} pump.

The role of mitochondria in the regulation of Ca^{2+} in smooth muscle is currently believed to be limited to slow processes associated with pathological states, in particular anoxia or ischemia (see refs. 12 and 442).

b. THE SOURCES OF ACTIVATOR CALCIUM: EFFECTS OF CALCIUM ANTAGONISTS ON CONTRACTILE RESPONSES. Families of concentration-effect curves similar to those shown in fig. 2. have been reproduced in depolarized rat aorta treated with various calcium antagonists, such as nifedipine (fig. 3) (417). It has been proposed by Bolton (87) and by Van Breemen (1091) that depolarization of smooth muscle increases the cell membrane permeability to calcium by opening membrane potential-dependent calcium channels. The first indication of the presence of Ca^{2+} channels in pericellular membranes was found by Fatt and Katz in 1953 (267) during a study of action potentials in crustacean muscle fibers. This observation was made just after the Na^+ theory of conduction of excitation in nerve had been proposed by Hodgkin and Huxley in 1952 (524). Among the observations was the capability of the muscle fibers to produce all-or-none action potentials when external Na^+ was replaced by tetraethylammonium or tetrabutylammonium. As shown by Sperelakis and coworkers (484, 1017), a similar observation can be made in vascular smooth muscle, where oscillatory calcium spikes can be produced when 12 mM tetraethylammonium is added to the bathing fluid. In such conditions, there is a blockade of the rectifying outwardly oriented K^+ current, and an inwardly directed current can be demonstrated. This electrical activity attributed to calcium entry can be blocked by agents identified as calcium antagonists (e.g., 781, 1017).

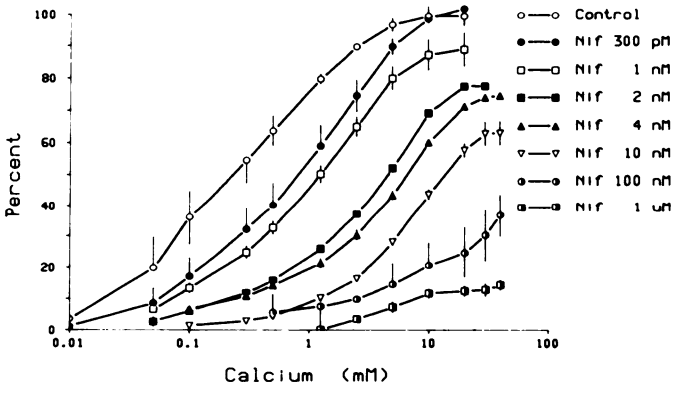

FIG. 3. Effect of nifedipine on contractions evoked by Ca^{2+} in K^+-depolarized rat aorta. Cumulative concentration-effect curves were obtained before and after addition of nifedipine (Nif) at the concentrations indicated. Responses are expressed as the percentage of maximal contraction evoked in the absence of nifedipine. Reproduced from Godfraind (402).

It has been shown that, when a rat aorta preparation has been preincubated in a calcium-free solution for sufficient time to abolish the response elicited by KCl-depolarizing solutions (usually 10 min), norepinephrine can still evoke a contraction, although weaker than that observed in normal physiological solution. This differentiates between a response that is directly dependent upon a concentration-dependent calcium entry from the extracellular space and a response that can be attributed to the release of calcium from a pool localized within the cell (418). Agents such as cinnarizine, flunarizine, and nifedipine that inhibit contractions evoked by adding calcium to calcium-free K^+-depolarizing solutions attenuate that part of the contractile response to norepinephrine directly dependent on extracellular calcium, whereas the response evoked in the absence of extracellular calcium is unaffected. The relative importance of the two components of the contractile response to stimulation of alpha-adrenoceptors depends not only on the concentration of calcium in the extracellular solution, but also upon the stimulating agent and on tissue factors extrinsic to the smooth muscle cell. The contractile response evoked by 10 μM norepinephrine (a maximum concentration) after pretreating the preparations with a maximally effective concentration of diphenylpiperazines, dihydropyridines, or verapamil derivatives is slightly higher than the contraction observed in Ca^{2+}-free solution (428). If it is assumed that the response resistant to calcium antagonists is only supported by intracellular calcium release, a quantitative estimate of the two components of the alpha-adrenergic contraction seems feasible. It will be shown later that studies of ^{45}Ca fluxes have identified an influx of Ca^{2+} resistant to those drugs, indicating that this statement is not completely correct, but that it can be accepted as a first approximation. However, high concentrations of diltiazem, for example close to 1 mM, block completely the response to norepinephrine, not only in rat aorta, but also in rabbit aorta. Ishizuka and Endo (557) and Saida and Van Breemen (944, 945) have shown that high concentrations of diltiazem inhibit, in skinned fibers, calcium release from intracellular calcium stores that are likely to be located in the sarcoplasmic reticulum. This effect could contribute to the attenuation of the vessel's response to catecholamines (see also section III, D and E).

Evidence has been presented for the presence of both $alpha_1$- and $alpha_2$-adrenoceptors subtypes in the plasma membrane of vascular smooth muscle (210, 1100), stimulation of both types by norepinephrine mediating vasoconstriction. In the pithed rat, Van Meel et al. (1100) have shown that pressor responses to $alpha_1$-adrenoceptor-selective agonists such as phenylephrine and methoxamine were not profoundly affected by pretreatment with nifedipine, but by contrast, the response to $alpha_2$-selective agonists such as azepexol hydrochloride (B-HT 933) and others was attenuated in a dose-dependent manner. These observations have been confirmed using other selective agonists and antagonists (e.g., 138, 942). In isolated rat aorta, the order of potency of sympathomimetic agonists is norepinephrine > phenylephrine > clonidine > oxymetazoline (431). If only $alpha_1$-adrenoceptors were present in the rat aorta, the expected order of potency of the alpha-adrenoceptor agonists should be phenylephrine > norepinephrine > oxymetazoline > clonidine. The reverse order might be expected if only $alpha_2$-adrenoceptors were present. The order of potency observed indicates that there could be a mixture of at least two alpha-adrenoceptor subtypes. Such observations are also consistent with the suggestion that the alpha-adrenoceptors of rat aorta are of $alpha_1$ subtype but different from $alpha_1$-adrenoceptors found in guinea pig and rabbit aorta, these last two most definitely being regarded as "typical" postjunctional $alpha_1$- adrenoceptors since they closely resemble those found in a variety of tissues (59, 937). However, binding experiments have demonstrated two distinct binding sites for prazosin and yohimbine in rat aorta (210).

In calcium-free solution, contractions stimulated by oxymetazoline and clonidine are almost abolished, while those stimulated by norepinephrine and phenylephrine are reduced by about 50%. As reported by Godfraind et al. (431), cinnarizine almost completely inhibited responses to oxymetazoline and clonidine but reduced norepinephrine- and phenylephrine-stimulated responses to such a degree that the residual contractions were close to those found in calcium-free solutions. This indicates that some sympathomimetics stimulate contractions that are almost totally dependent on extracellular calcium. Others stimulate contractions that are partly dependent on extracellular calcium and partly dependent on intracellular calcium stores.

The calcium dependency of the contractile response of rat isolated aorta to various agonists appears to be modulated by the endothelium. The essential role of endothelium as a mediator of relaxant responses induced in isolated vascular tissues by acetylcholine and other vasodilators is now well established (357, 358). Its role as a modulator of contractile effects of agonists in some vascular smooth muscles also seems to be important. Indeed, removal of endothelium evokes a dramatic effect on the contractile responsiveness of rat aorta to clonidine (128a, 239): the concentration-effect curve for clonidine is shifted to the left and the maximum tension developed shows a 9-fold increase. An increase in sensitivity after endothelium removal has been observed not only with $alpha_2$-adrenoceptor agonists that have a weak effect in aortas with endothelium, but also with full $alpha_1$-adrenoceptor agonists (128a, 239, 411, 700). This increase of responsiveness evoked in rat aorta by endothelium removal is also found when the preparation is stimulated by serotonin, prostaglandin F_{2alpha} (PGF_{2alpha}), and the calcium agonist, Bay K 8644 (240, 411, 1015) and is associated with changes in the effect of calcium antagonists. Analysis of phenylephrine- and norepinephrine-

induced contractions in the absence and in the presence of the maximal effective concentrations of nifedipine and flunarizine shows that the amplification of the contraction in response to low concentrations of norepinephrine (10 nM or less) is very sensitive, whereas the responses to high doses of norepinephrine (0.1 μM and above) are perhaps more resistant to those calcium antagonists (412, 764). That is, in the absence of endothelium, calcium entry blockers are apparently more potent than in the presence of endothelium, and this effect has also been noted with PGF_{2alpha}- and Bay K 8644-induced contractions (424, 429, 764, 1015). These experiments show that important methodological problems may arise when testing calcium antagonists. In addition, they indicate that analysis of contractile responses does not necessarily provide information allowing a proper understanding of their effects. This understanding requires characterization of processes responsible for cellular calcium regulation.

c. EFFECTS OF CALCIUM ANTAGONISTS ON ^{45}Ca MOVEMENTS. The above observations show that several factors control or modulate the contractile responses of vessels. This emphasizes the need for direct study of calcium fluxes and of calcium channels. Direct measurements of ^{45}Ca movements are not useful in smooth muscle because they are not indicative of changes that could occur within the cell, partly due to the large amount of calcium that is bound to extracellular sites (421). Several attempts have been made to identify the biologically active calcium fraction, and the most successful are based on the use of lanthanum. La^{3+} replaces Ca^{2+} on superficial binding sites and does not penetrate vascular smooth muscle cells. Because lanthanum also blocks transmembrane fluxes of calcium, it has been proposed that the calcium content of a muscle washed in a lanthanum solution could provide an estimate of cellular calcium. Therefore, ^{45}Ca fluxes across the smooth muscle cell membrane may be estimated in the rat aorta by measuring the ^{45}Ca turnover in the La^{3+}-resistant Ca^{2+} fraction. This fraction corresponds to the amount of Ca^{2+} that is not displaced when the tissue is soaked in a 50 mM La^{3+}-containing solution (397). Norepinephrine increases the rate of uptake of ^{45}Ca into this calcium fraction in a concentration-dependent manner (397). There is no net gain in tissue calcium and calcium efflux increases in a similar manner. In the presence of the alpha-adrenoceptor antagonist, phentolamine, the concentration-response curves for norepinephrine on ^{45}Ca uptake are displaced to the right in a manner suggesting a competitive antagonism, the pA_2 for phentolamine being equal to 7.8 (410). Because of the resemblance of this pA_2 value to that obtained for the antagonistic effects of phentolamine on the contractile responses to norepinephrine (396, 410), it appears that the activation of alpha-adrenoceptors is responsible for both the increased rate of calcium entry and contraction. The observations suggest that alpha-adrenoceptor activation does open Ca^{2+} pathways in the membrane, which have been termed receptor-operated channels (ROCs) (87). Exposure to high-K^+ solutions also results in an increase in the influx of ^{45}Ca that presents some differences from agonist-stimulated influx, namely, as discussed below, its sensitivity to calcium antagonists. Those channels controlled by membrane potential have been termed potential-operated channels (POCs). In addition to ROCs and POCs, Bevan et al. (69) have proposed the existence in some vessels, mainly in cerebral arteries, of stretch-operated channels (SOCs). The slow turnover of intracellular calcium at rest is likely to occur through Ca^{2+} leak channels, although an exchange process or a type of transport system cannot be excluded.

Most of the experimental observations accumulated to date indicate that inhibition of contraction by diphenylpiperazines, dihydropyridines, diltiazem, and verapamil is related to blockade of calcium entry through calcium channels operated by receptors and membrane potential. Blockade of stimulated calcium influx has similar time-dependent characteristics as blockade of contraction. Concentration-effect curves for inhibition of contraction and Ca^{2+} influx by flunarizine, cinnarizine, and nifedipine are very similar in rat aorta (402, 410, 427), suggesting that reduction of norepinephrine effects on muscle tone is due to inhibition of agonist-dependent ^{45}Ca entry specifically sensitive to the blockers. ^{45}Ca entry into a resting smooth muscle is reduced only in the presence of large concentrations of the blockers (close to the concentration producing a maximum effect on the contraction), and even then the effect is small (157, 410, 428, 431). As fig. 4 illustrates, in the presence of a maximum effective concentration of several calcium entry blockers, part of the norepinephrine-dependent influx (10 to 15%) is resistant to blockade. This indicates that contractions resistant to calcium entry blockers may be supported by this resistant calcium influx. However, in view of the presence of a large contraction in calcium-free medium, it is most likely that intracellular calcium also contributes to the support of contractions resistant to calcium entry blockers. This is confirmed by the observation that norepinephrine evokes a release of calcium from arteries in calcium-free solution, which is resistant to nifedipine and related drugs. Fig. 4 also shows that the release of calcium evoked in physiological solution is barely affected by the calcium entry blockers.

When calcium is present in the perfusion fluid, ^{45}Ca efflux from arteries preloaded in radioactive solution can also be observed. This efflux appears to be due to displacement of ^{45}Ca bound to intracellular sites by ^{40}Ca. Depolarization in K^+-containing solution does not seem to release intracellular calcium unless calcium is present in the bathing medium. This reinforces the idea that K^+-induced contractions are dependent on an entry of extracellular Ca^{2+}, and these contractions and the evoked ^{45}Ca entry can be completely blocked by nifedipine, flunarizine, and related drugs. Not surprisingly, concentrations

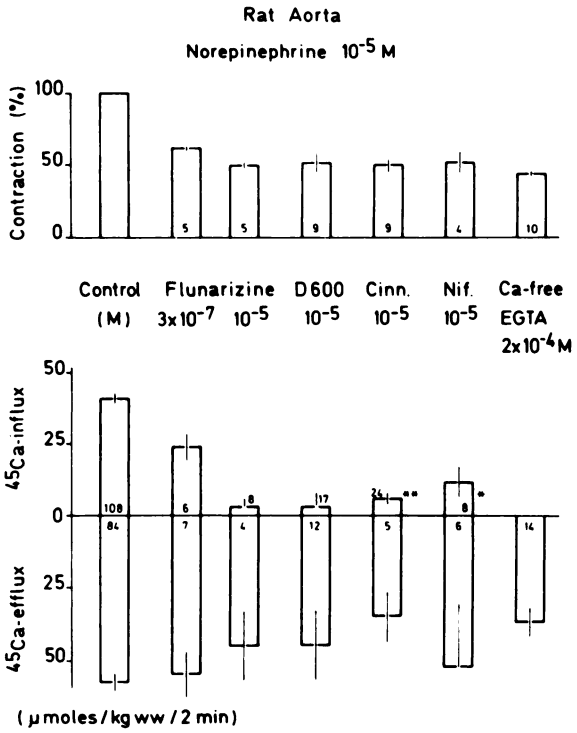

FIG. 4. Effect of calcium entry blockers and of Ca^{2+}-free medium on contraction (*top*) and ^{45}Ca influx and efflux (*bottom*) evoked by norepinephrine (10 μM) in rat isolated aorta. Tissues were preincubated with blockers for 90 min, or with EGTA for 5 min, before addition of norepinephrine. Contractions are expressed as the percentage of the contraction induced in the absence of drug (control). *Numbers in columns*, number of determinations; *vertical bars*, SE. *Cinn.*, cinnarizine; *Nif.*, nifedipine. Reproduced from Godfraind and Miller (428).

of the blockers sufficient to abolish ^{45}Ca entry also abolish ^{45}Ca efflux.

When ^{45}Ca entry is measured at various times following K^+ depolarization, the amount of ^{45}Ca entering the cell for a given unit of time declines with the duration of the K^+ depolarization. For instance, after a depolarization of 30 min, ^{45}Ca entry, expressed in $\mu mol \cdot kg^{-1} \cdot min^{-1}$, is about two-thirds of the amount measured after a K^+ depolarization of 2 min. This could indicate inactivation of the channels. The sensitivity of this influx to blockade by nisoldipine and flunarizine, estimated after 2- and 35-min duration of the depolarization, was obviously greater after 35 min (408). As shown in other reports on flunarizine and cinnarizine, this increase in sensitivity of ^{45}Ca entry blockade is associated with an increase in the pharmacological action, since there is a shift to the left of concentration-inhibition curves when the inhibition of the contraction is measured at different times after the commencement of depolarization (410, 425, 426).

2. Differences between rat and rabbit aortas. The aortas of the rat and the rabbit are much used as models of vascular smooth muscle reactivity. Contractions induced by K^+ are inhibited completely by a short (min) preincubation period in calcium-free solution in both vessels and are, therefore, thought to be totally dependent on extracellular calcium (see section II A 1). These contractions are inhibited by calcium entry blockers (table 2), and the inhibition has been correlated with an inhibition of ^{45}Ca influx (135, 137, 384, 402, 410, 431, 432, 525, 603, 1093, 1151). Inhibition of K^+-stimulated ^{45}Ca uptake by D 600 and nitrendipine in cultured cells derived from rat aortic smooth muscle has also been reported (652).

The group of calcium entry blockers derived from dihydropyridine are the most potent as inhibitors of K^+-induced contractions, having 50% inhibitory concentration (IC_{50}) values in the nM range. The collected data in table 2 in general show no great differences between these derivatives as far as potency is concerned. From experiments directly comparing dihydropyridines in rabbit aorta (21, 896, 1065), a potency order of nicardipine > nitrendipine > nifedipine can be deduced, the overall difference in potency being about 4-fold. In rat aorta, the collected data indicate that perhaps nimodipine is more potent than nifedipine and that nisoldipine is the most potent. Where calcium entry blockers from various chemical classes have been directly compared, there is a potency order of dihydropyridine > verapamil > diltiazem in both vessels (21, 289, 1018, 1169) with a ratio between diltiazem and verapamil of between 3 and 6.

It is interesting that complete inhibition of K^+-induced contractions of rabbit aorta has not always been reported with dihydropyridines (table 2) (289, 513, 1068) or with bepridil, verapamil, or diltiazem (289, 840, 1094). Incomplete blockade of K^+-induced contractions in rat aorta has not been reported. The reason for this difference is not known but might indicate a small role for intracellular calcium in responses of rabbit aorta, perhaps activated by depolarization-induced liberation of norepinephrine from sympathetic nerve endings. Other possible explanations are a role for another calcium entry mechanism such as Na^+-Ca^{2+} exchange, the existence of a small subpopulation of channels not sensitive to the blockers, or, of course, a difference between the calcium channels in the two tissues assuming homogeneous channel populations. This latter possibility would seem to be unlikely on functional grounds, since there is no obvious difference in the potency of the various calcium entry blockers in the two tissues (table 2).

The two vessels differ in several important respects where activation by agonists is concerned. Norepinephrine-induced contractions of both arteries are only partly dependent on extracellular calcium. After washing in calcium-free, EGTA-containing, physiological solution for about 10 to 15 min, a procedure that completely inhibits K^+-induced contractions in both arteries (110, 129, 418, 1092, 1094), norepinephrine-induced contractions are inhibited by 50 to 60% in rat aorta (above) and by 20 to 30% in the rabbit vessel (1092). As might be expected, in normal physiological solution norepinephrine-induced contractions of the rat aorta are inhibited by calcium entry blockers by about 40 to 60%. In the rabbit, entry blockers have slight (up to 30% but more

TABLE 2

IC_{50} values of calcium entry blockers in rabbit and rat aorta as inhibitors of agonist- and depolarization (K^+)-induced contractions

Blocker	K^+ IC_{50} (nM)	Agonist*	IC_{50} (nM)	Maximal inhibition of agonist (%)	Ref.
Rabbit aorta					
FR 34235	1				(359)
Nitrendipine	4.7†				(21)
Nitrendipine	30	Nor		30	(1148)
Nitrendipine	3.1				(1065)
Nifedipine	4				(359)
Nifedipine	1.4	Nor		25	(896)
Nifedipine	11.2				(974)
Nifedipine	<100‡	Nor		6 (NS)	(289)
Nifedipine		Nor		0	(513)
Nifedipine	7.8				(1018)
Nifedipine	8.1				(1065)
Nifedipine	30				(969)
Felodipine		Nor		0	(690)
Nisoldipine	8.7†				(21)
Nicardipine	0.32	Nor		25	(896)
Nimodipine	5	Nor		0	(1065)
Nimodipine	2.9				(623)
(±)Nimodipine	5.8‡	Nor		0	(1068)
(+)Nimodipine	16‡				(1068)
(−)Nimodipine	3‡				(1068)
Verapamil		Nor		10	(526)
Verapamil		5HT		15	(526)
Verapamil		Angio		20	(526)
Verapamil		Nor		12	(722)
Verapamil	1,000				(782)
Verapamil	500	Nor		25	(896)
Verapamil	69.3				(21)
Verapamil	150	Nor	>10,000	55	(605)
Verapamil	170‡	Nor		19 (NS)	(289)
Verapamil	100‡			0	(840)
Verapamil	300				(1018)
Verapamil	140				(1065)
Verapamil	68	Nor	>10,000		(603)
D 600	100				(1095)
D 600	26.9§				(974)
Diltiazem	1,000‡	Nor		8 (NS)	(289)
Diltiazem	222†				(21)
Diltiazem	500	Nor		25	(135)
Diltiazem	1,200				(1018)
Diltiazem	500†	Nor	500†		(137)
PY 108-068		5HT		20	(526)
PY 108-068		Angio		20	(526)
PY 108-068		Nor		0	(526)
Bencyclane	3,000				(623)
Bepridil	3,000				(782)
Bepridil	1,000‡	Nor		22	(289)
Lidoflazine	20,000	Nor		13	(1083)
Lidoflazine	20,000	Angio		7	(1083)
Lidoflazine	4,000	5HT		53	(1083)
Papaverine	12,000				(896)
Papaverine	26,300				(974)
Perhexiline	3,200			0	(840)
Rat aorta					
Nifedipine	5				(755)
Nifedipine	2.6				(1166)
Nifedipine	1.3				(441)
Nifedipine	1	Phe	1		(958)
Nifedipine	1	Nor	<1		(958)
(−)Nimodipine	0.2	PGF_{2a}	0.73	>30	(441)
(+)Nimodipine	1.4	PGF_{2a}	14		(441)
(±)Nimodipine		PGF_{2a}	2.5		(441)
Nisoldipine	0.03	Nor	0.82		(412)
Caroverine	240	Nor			(552)
Verapamil	66				(552)
Verapamil	76.1				(1166)
Verapamil	100				(603)
D 600		Clon	10	100	(822)
D 600		Nor	23	50‖	(822)
Diltiazem	207				(1166)
Cyproheptadine	63				(1166)
Cinnarizine	38	Nor	500	60	(431)
Cinnarizine		Phe	240	64	(431)
Cinnarizine		Clon	550	92	(431)
Cinnarizine		Oxy	240	93	(431)
Cinnarizine		Nor		73	(460)
Corynanthine	28,000	PGF_{2a}		0	(432)
Rauwolscine	27,000	PGF_{2a}	15,000	8	(432)

* Abbreviations: Nor, norepinephrine; 5HT, serotonin; Angio, angiotensin; Phe, phenylephrine; PGF_{2a}, prostaglandin F_{2alpha}; Clon, clonidine; Oxy, oxymetazoline; NS, inhibition not significant.

† IC_{50} for inhibition of ^{45}Ca influx.

‡ Inhibition of K^+ contraction was incomplete.

§ Calculated from pD_2 values.

‖ One μM gallopamil (D 600) inhibited norepinephrine (10 μM) contractions by about 50%. A 1 mM concentration of D 600 almost completely inhibited these norepinephrine-induced contractions.

usually about 15 to 20% inhibition) or no antagonistic effect on norepinephrine-induced contractions (table 2). Norepinephrine-stimulated ^{45}Ca influx is attenuated to a large extent by calcium entry blockers in rat, but not rabbit, aorta (288, 402, 428, 431, 432).

This considerable variability in the maximal degrees of inhibition reported could be due to several possible contributing factors. Time of preincubation with blockers may play a part, since some [class 3 of Spedding (1008, 1012)] reach equilibrium with the tissues only after prolonged preincubation times, for example, about 90 min in the case of bepridil, cinnarizine, and flunarizine, that are not always a feature of experimental protocols. The use of concentrations of blockers greater than those known to be necessary to inhibit calcium entry, or the use of blockers without any verification of the concentration range over which they can be expected to inhibit calcium entry, will probably result in effects of these compounds that are unrelated to calcium entry blockade. For example, 0.1 mM cinnarizine will inhibit norepinephrine-induced contractions of rat aorta by about 90% (823), 0.2 mM diltiazem by about 80% (460), and 1 mM D 600 will almost abolish them (822). At a concentration of 3 μM, cinnarizine will practically abolish agonist-stimulated ^{45}Ca influx in this tissue (431), where

it has an IC_{50} value of about 38 nM (table 2), and a 10 μM concentration of flunarizine, D 600, and nifedipine (fig. 4) is maximally effective. Large concentrations (0.1 mM) of cinnarizine (also fendiline and flunarizine) probably have intracellular effects (1010), perhaps by an interaction with calmodulin (section III C). However, 0.1 mM verapamil does not seem to affect the contractile apparatus (1010), so the inhibitory effect of large concentrations of D 600 might be explained by alpha-adrenoceptor antagonism (section III D).

A further factor perhaps responsible for some of the variability of calcium entry blocker effectiveness may be due to an effect of the endothelium on the action of the blockers. It has recently been shown that flunarizine and nifedipine are more effective as antagonists of agonist-induced contractions in the absence than in the presence of endothelium (412, 764). Therefore, differences in the manner of preparation of aortic tissues, resulting in the presence of variable amounts of endothelium, might be expected to produce variable results.

A more prolonged exposure to calcium-free solution containing a calcium chelator (about 20 to 30 min), that will abolish norepinephrine-induced contractions in rat aorta (418), depresses contractions in the rabbit vessel only slightly more than the shorter treatment in calcium-free solution. Evidently, intracellular calcium stores are much more important in the rabbit aorta for norepinephrine-induced responses than they are in the rat aorta. Again, very large concentrations of verapamil or diltiazem will produce more marked inhibitory effects (576, 1094). In studies to directly compare calcium entry blockers in rabbit aorta, a similar maximal degree of inhibition by nicardipine, nifedipine, and verapamil has been found (896), although it has been reported that bepridil inhibited norepinephrine-induced contractions and ^{45}Ca influx, while verapamil, nifedipine, and diltiazem were inactive (289). However, in this latter study, the inhibition by bepridil and verapamil was of the same order (22 and 19%, respectively).

It has generally been thought that, in the rat vessel, Ca^{2+} enters the cytoplasm subsequent to agonist stimulation by two means: liberation from an intracellular store, probably the sarcoplasmic reticulum; and translocation from the extracellular space via specific calcium channels in the sarcolemma. In the case of the rabbit vascular smooth muscle, evidence has been advanced suggesting that all calcium for contraction that enters the cytoplasm does so via the intracellular calcium store (129, 130). That is, there is no direct connection between the extracellular space and the cytoplasm activated by norepinephrine. This calcium entry into an intracellular store is effected by an unknown mechanism that is not sensitive to calcium entry blockers, even on prolonged (up to 90 min) preincubation (157, 410, 428, 552, 562). This has been called the leak channel and is apparently inhibited by low concentrations (1.5 mM) of lanthanum because, in the presence of lanthanum, the ^{45}Ca space (453) is about equal to the extracellular space (121). Other trivalent ions of the lanthanide series have similar effects (219), and divalent ions such as Cd^{2+} and perhaps Zn^{2+} (177) are also inhibitors, but Mg^{2+} is only a weak inhibitor (601).

If it is the case that there are no, or comparatively few, calcium channels activated by agonists in rabbit aorta, and that calcium entry blockers do not affect the leak channel, then it is not surprising that the blockers have little or no effect on maximal agonist-induced responses. However, when relatively low concentrations of norepinephrine (producing about 30% of the maximal response) are used to stimulate rabbit aorta preparations, diltiazem is an effective inhibitor of the stimulated ^{45}Ca entry with an IC_{50} value (about 0.1 μM) similar to that found against K^+-induced contractions (137, 1094). In these experiments, norepinephrine did not depolarize the smooth muscle cell membrane (136, 137). Therefore, it seems that, in the rabbit aorta, low concentrations of norepinephrine stimulate the opening of receptor-operated channels that are responsible for up to 20 to 30% of the maximal contractile response. High concentrations elicit contractions largely dependent on intracellular calcium and insensitive to calcium entry blockers. The replenishment of the intracellular calcium stores is dependent on Ca^{2+} entry into the cell via a mechanism that is not a calcium channel, or at least not a channel sensitive to calcium entry blockers.

When agonists other than norepinephrine are considered, their sensitivity to blockers will depend to a great extent on their relative dependence on intra- or extracellular calcium to produce contractions. This is considered in section III F.

3. Cerebral arteries. a. CAT. After 20 min in Ca^{2+}-free (10 μM EGTA) solution, PGF_{2alpha} (20 μM) elicited biphasic contractions in cat basilar artery preparations of about 87% of those elicited in normal Ca^{2+}-containing solution. These contractions were inhibited by Mn^{2+} and diltiazem. Both were more effective as antagonists of the second phase of the PGF_{2alpha} contraction than of the first phase. Diltiazem and nifedipine (10 μM) abolished the second phase and reduced the first phase by about 60 or 87%, respectively. Bay K 8644 could restore the second phase in the presence of nifedipine but not of diltiazem (1088). These observations tend to suggest an extracellular source of Ca^{2+}, and the effect of the Ca^{2+} withdrawal treatment on K^+-induced contractions was not tested. However, pretreatment with 20 mM caffeine almost abolished PGF_{2alpha}-induced contractile activity (1086, 1087), apparently indicating that a major part of the calcium mobilized by PGF_{2alpha} is of intracellular origin. In the cat middle cerebral artery, 30 min in Ca^{2+}-free solution reduced K^+ (60 mM) contractions to about 8% of control. This small residual contraction was inhibited, but not abolished, by 0.3 and 0.21 μM, respectively, of nifedipine and nimodipine (22). In another series of experiments, 10-min exposure to Ca^{2+}-free solution vir-

tually abolished K^+ (124 mM)-induced contractions, but nifedipine maximally relaxed K^+-contracted arteries by only 87%, IC_{50} 3.3 nM (993). Verapamil (IC_{50} 0.11 μM) was significantly more effective at relaxing contracted cat basilar arteries than was diltiazem (IC_{50} 0.6 μM) (1165). Preincubation in Ca^{2+}-free physiological solution for 10 min was sufficient to abolish both phases of norepinephrine-induced contractions in cat middle cerebral arteries (993).

b. DOG. Withdrawal of Ca^{2+} (0.1 mM EGTA) for 10 min was sufficient to abolish K^+- and 15-hydroperoxyarachidonic acid (15-HPAA)-induced contractions of dog basilar artery (33) as was treatment with 2 mM EGTA (355), although after 60 min in Ca^{2+}-free solution, carbocyclic TxA_2 ($cTxA_2$, a stable analogue of thromboxane A_2)-mediated contractions of basilar and middle cerebral arteries were reduced by only about 51% (1062). In these arteries, $cTxA_2$ contractions were maximally relaxed by 74% by 10 μM verapamil, IC_{50} 0.1 μM, compared to a maximal papaverine-induced relaxation. The discrepancy between that part of the contraction resistant to removal of Ca^{2+} and the relaxant effect of verapamil may be accounted for by an effect of the drug unrelated to blockade of calcium entry. In dog basilar artery, Ca^{2+}- and 15-HPAA-induced concentration-effect curves were shifted to the right, and the maximum contraction was depressed by 40 nM and 0.2 μM verapamil, 0.01 to 1 nM PN 200-110, 3 to 30 nM nifedipine, and 0.3 to 3 nM nimodipine (33, 789). K^+- but not caffeine-induced contractions were suppressed by diltiazem concentrations greater than 0.3 μM (355), and maximal serotonin contractions were about 25% resistant to 30 nM nifedipine when arteries were preincubated (5 min) with the blocker, although arteries precontracted with serotonin were relaxed completely by the same concentration, and maximal phenylephrine contractions were about 12% resistant (15). The nicardipine IC_{50} value as an antagonist of serotonin contractions was about 25 nM (806). After 30-min preincubation with PN 200-110, nifedipine, or nimodipine, serotonin concentration-effect curves were antagonized noncompetitively, but about 20% of the maximal serotonin-induced contraction was not abolished by 1 μM nifedipine (789).

c. HUMAN. K^+ contractions of human pial vessels were reduced to only 30% of normal after 30 min in Ca^{2+}-free solution as were norepinephrine- and serotonin-induced contractions (102). Preincubation for 10 min with nifedipine (0.1 μg/ml; 0.29 μM) before eliciting concentration-effect curves inhibited maximal norepinephrine- and serotonin-induced contractions by about 83 and 60% and shifted the curves to the right. If added to precontracted arteries, nifedipine produced an almost complete relaxation (238). Also, Ca^{2+} concentration-effect curves elicited in human anterior cerebral arteries in the presence of 60 mM K^+ were displaced to the right, and the maximal responses were inhibited by PN 200-110, nifedipine, or nimodipine. Calculated from the contractions elicited by 1.6 mM Ca^{2+}, IC_{50} values were, respectively, 33 pM, 2.2 nM, and 0.3 nM, but the maximal concentrations of blockers tested (1 nM, 30 nM, and 3 nM, respectively) did not abolish contractile activity. Blood-induced vasospasms were also inhibited, but not abolished (about 17% resistant) by PN 200-110, nifedipine, and nimodipine, IC_{50} values 1.9 nM, 10 nM, and 1.6 nM, respectively (789). In middle cerebral arteries, K^+ (75 mM)- and serotonin (10 μM)-induced contractions were completely relaxed by 10 μM verapamil, but about 10% of a 10 μM norepinephrine contraction was resistant to the antagonist. IC_{50} values of verapamil were, respectively, 0.12, 0.31, and 0.72 μM (950).

d. GOAT. Goat cerebral arteries, studied in parallel with the above-described human middle cerebral arteries, when contracted by K^+ or serotonin were completely relaxed by 10 μM verapamil, but norepinephrine contractions were about 15% resistant. The IC_{50} verapamil values were, respectively, 0.1, 0.95, and 1.78 μM (950). The parallel between the responses in the vessels from the two species is quite strong.

e. RAT. Contractions of rat basilar and middle cerebral arteries in K^+-rich solutions, that were abolished by La^{3+}, IC_{50} about 3 μM (531), but only reduced by about 90% by 10-min incubation in Ca^{2+}-free solution (993), could be resolved into three components in the presence of nifedipine. The tonic contraction was selectively inhibited by nifedipine (IC_{50} about 7 nM for tonic, about 70 nM for phasic), diltiazem, verapamil, flunarizine, and felodipine. However, nifedipine inhibition was incomplete (about 16% of tonic and 43% of phasic resistant to inhibition), as was inhibition of Ca^{2+} concentration-effect curves (531). Sustained norepinephrine-induced contractions were inhibited by about 80% after 10 min in Ca^{2+}-free solution and inhibited by 77% by nifedipine, IC_{50} 10 nM, in normal solution (993).

f. MOUSE. Ba^{2+}-induced contraction of mouse pial arterioles in situ was inhibited by verapamil, IC_{50} about 0.2 μM, but 100% inhibition of contraction was not demonstrated (928). In this model the maximal inhibition of norepinephrine-induced reduction in diameter was about 60 to 70% by verapamil, nisoldipine, and nimodipine administered i.p. PGF_{2alpha}- and serotonin-induced contractions seemed to have a similar degree of resistance to the blockers (927).

g. RABBIT. K^+-induced contractions of rabbit basilar artery were not abolished by 30 μM nifedipine (20% resistant, IC_{50} about 10 nM) in one report (532), and the nimodipine IC_{50} value was about 8 nM (1066), although they were almost abolished by 10 nM FR 34235 (1044). In other studies they were abolished by nifedipine, nicardipine, bencyclane, and verapamil with IC_{50} values of 1.1 nM, 12 nM, 2.6 μM, and 29 nM, respectively (806), and by nimodipine, IC_{50} 24 nM (1064). Electrically stimulated contractions were 100% inhibited by nifedipine, and a relatively high concentration of nicardipine (IC_{50} about 0.8 μM) (806). Serotonin contractions of rabbit

basilar artery were biphasic, and both phases were depressed in a nominally Ca^{2+}-free physiological solution, but the tonic contraction was abolished more quickly than the phasic contraction, suggesting that limited intracellular Ca^{2+} stores might be mobilized by serotonin. Nimodipine selectively inhibited the second tonic phase with an IC_{50} of 0.73 nM. The phasic component of contraction was inhibited by 10 nM to 10 μM nimodipine (1064). Readmission of Ca^{2+} to arteries exposed to serotonin in nominally Ca^{2+}-free solution induced contractions that were abolished by nimodipine (1064). Serotonin-induced concentration-effect curves were depressed by about 50% by 10 nM FR 34235, nimodipine, and nifedipine, but this concentration of nimodipine had no inhibitory effect on the small (532) norepinephrine-induced responses (1044). The nimodipine IC_{50} value was about 1.1 nM against $cTxA_2$-induced contractions but, compared to maximal papaverine-induced relaxations, about 15% of the contraction was resistant to nimodipine (1067). Nifedipine depressed serotonin (0.25 μM) contractions by only about 24% (623).

h. GUINEA PIG. In guinea pig basilar arteries (diameter, about 200 to 300 μm), nicardipine at a concentration of up to 3 μM did not affect the resting membrane potential of 50.1 ± 1.1 mV, while 1 μM had a small inhibitory effect on excitatory junction potentials (inversely related to pulse number, i.e., the reverse of the "use-dependent" effects usually seen in cardiac tissue, for example) elicited by perivascular nerve stimulation and little or no effect on associated contractile activity (356). In this vessel, contractions induced by K^+ (77.2 mM), ATP, and exposure to Na^+-free solution were abolished in Ca^{2+}-free medium, and serotonin contractions were reduced to about 10% of those in normal physiological solution. At a concentration of 1 μM, nicardipine virtually abolished K^+-induced responses, partially inhibited ATP responses (by about 40%), and had no effect on serotonin- or caffeine-induced contractions. At the concentrations tested (1 μM for serotonin and 1 mM for ATP), ATP but not serotonin effects were associated with a reduction in membrane potential (356). These observations seem to indicate that nicardipine inhibits selectively a potential-dependent entry of extracellular calcium and might be considered as evidence for the existence of two channel types. However, the pretreatment time with nicardipine before testing of responses was not specified, and short incubation times can give an exaggerated idea of selectivity of antagonism of responses (see below section III F).

i. CONCLUSION. The potency of calcium entry blockers as antagonists of K^+- or agonist-induced contractions in cerebral arteries does not differ greatly overall from data obtained in large vessels, such as the rat and rabbit aorta. Marked differences are seen in that frequently a component of contraction is resistant to inhibition. This has sometimes, but not always, been ascribed to release of intracellular Ca^{2+}. The presence of calcium channels relatively insensitive to known calcium entry blockers might be inferred. The resistance of K^+-induced contractions to the blockers may be due to the evoked release of a neurotransmitter capable of mobilizing intracellular Ca^{2+}, and attention should be paid to this possibility. Some calcium entry blockers show a predilective action on cerebral arteries (981), a property that requires further investigations.

4. Coronary arteries. Depolarization of human, dog, pig, and bovine coronary arteries with or without spontaneous activity (see below) stimulates an influx of calcium (523) and produces a stable contraction that can be inhibited by diltiazem, lidoflazine, nifedipine, verapamil proadifen (SKF 525A), and FR 7534 (24, 311, 382, 409, 466, 867, 1095, 1130). Godfraind et al. (427) found that, while nifedipine, lidoflazine, and flunarizine completely inhibited such contractions in arteries derived from older human hearts, contractions of arteries from younger hearts were partially resistant to the calcium entry blocking compounds. Older arteries were also more sensitive to the calcium entry blocking drugs, the most marked difference being seen with nifedipine, which was 100-fold more potent in older than in younger arteries. Flunarizine potency increased by about 10-fold, and that of lidoflazine hardly at all (428). These changes perhaps indicate a change in reactivity with increasing age or pathological condition, that may in part be related to changes in the endothelium (see section II A 1). A component of depolarization-induced contractions of rabbit, pig, and human coronary arteries, dependent on extracellular calcium but resistant to nifedipine, has also been described (443, 445) and, while Fleckenstein (299) described depolarization-induced contractions of pig coronary arteries that were completely inhibited by nifedipine (IC_{50} about 7.6 nM) and verapamil (IC_{50} about 0.1 μM), about 15% of the contracture seemed to be resistant to diltiazem (IC_{50} about 0.2 μM). K^+-contracted dog coronary arteries were only inhibited by about 86.5% by lidoflazine (IC_{50} 1 μM) (1106). Nifedipine, verapamil, and tiapamil also depressed these contractions (IC_{50} 9 nM, 0.17 μM, and 0.22 μM, respectively) (244, 1130), and they were abolished by D 600 (IC_{50} 0.1 μM) and SKF 525A (IC_{50} 3 μM) (1095). Inhibition by lidoflazine was partially reversed by doubling the calcium concentration (1106), but cumulative additions of calcium to arteries inhibited by nifedipine, verapamil, and tiapamil produced varying results. Verapamil depression was reversed by 94%, that of tiapamil by 59%, and that of nifedipine by 27%. The interactions of these compounds with the cell membranes of the coronary arteries do not therefore appear to be identical, even though in depolarized rat renal arteries (244) calcium concentration-effect curves were apparently antagonized competitively by tiapamil and verapamil, their pA_{10} values being 6.5 and 7.1, respectively, and pA_2 values for nifedipine and verapamil have been determined in intestinal smooth muscle (table 5).

These observations indicate a degree of variability in

the efficacy of calcium entry blocking drugs in the coronary vasculature similar to that seen between other vascular beds and are probably an indication of the heterogenous nature of potential-sensitive calcium channels (478).

a. CALCIUM. Calcium in concentrations up to about 8 mM, in the absence of depolarization, induced contractions of beef and pig coronary arteries that were antagonized by verapamil, D 600, prenylamine, and nifedipine (470). Calcium-dependent action potentials in dog coronary artery smooth muscle could be elicited in the presence of tetraethylammonium (TEA), and they were inhibited by bepridil (IC_{50} 3 μM) and partially restored by increasing the calcium concentration of the bathing solution (485). Verapamil also depressed these action potentials (60).

Addition of digoxin or ouabain after TEA treatment mediated an increase in spontaneous oscillations in membrane potential in dog coronary arteries and these were abolished by verapamil (60). Cardiac glycosides induced contractions of beef and pig coronary arteries in normal physiological solutions (304, 932) that could be prevented or reversed by verapamil and D 600 (304). Contractions elicited by increasing the calcium concentration bathing pig coronary arteries were potentiated by cardiac glycosides, and these potentiated contractions were much less sensitive to verapamil, D 600, prenylamine, and nifedipine (470). Even though Na^+-Ca^{2+} exchange has very little, if any, role in reducing intracellular Ca^{2+} levels subsequent to contractile activity in normal vascular smooth muscle (773, 1091, 1092), this reduced potency of calcium entry blockers in the presence of cardiac glycoside-enhanced contraction can be explained if part of the mechanism of action of cardiac glycosides is to reduce Na^+ efflux, thereby increasing the influx of Ca^{2+} by a Na^+-Ca^{2+} exchange mechanism as occurs in cardiac muscle. Such an exchange would not be sensitive to calcium entry blockers.

b. SPONTANEOUS ACTIVITY. Various types of spontaneous rhythmic activity in isolated human coronary artery preparations have been described (382, 427, 428, 443, 660, 661, 929), but such phenomena are not always apparent (79, 179, 594, 617, 995, 996). Godfraind and Miller (427, 428) only noted spontaneous activity in some arterial segments from older hearts in which the arteries contained visible atherosclerotic plaques. In nonactive segments, taken from the same arteries, rhythmic activity could sometimes, but not always, be induced by histamine or serotonin (382) or PGF_{2alpha} (617). These spontaneous fluctuations in tone were inhibited in calcium-free physiological solution and by nifedipine (413, 443), as are induced rhythmic tone changes (see below).

The reason for the appearance of spontaneous contractile activity seen in in vitro preparations of human coronary arteries is unknown, but it could be a prelude to spasmodic activity in vivo. It is interesting that alterations in the cholesterol content of tissues change their responsiveness to stimuli and that these changes in responsiveness are linked to changes in calcium sensitivity. In low concentrations, cholesterol was incorporated into isolated coronary arteries of dogs (measured as uptake of ^{14}C label) and induced a slowly developing contraction. At the same time, the arteries became more sensitive to the contractile effects of changing extracellular concentrations of either Ca^{2+} of K^+. These cholesterol-induced contractions were sensitive to verapamil and, therefore, probably dependent on extracellular calcium. The mechanism of these actions was thought to be by induction of a change in membrane fluidity due to the incorporation of cholesterol (285, 1181). Removal of cholesterol from cell membranes with digitonin, a detergent which in low concentrations is used to specifically label membrane cholesterol (1158), increases their permeability to calcium (1167). Changes in the cholesterol content of cardiac myocytes also affect their functioning; a reduction in content enhances the rate of depolarization and increases their resistance to the effects of lowered Na^+ or Ca^{2+} levels and also their resistance to the effects of tetrodotoxin and verapamil (1024).

These results might indicate that increased incorporation of cholesterol into membranes renders them more permeable to calcium, which would tend to induce a contraction of smooth muscle. However, these observations do not necessarily imply that spontaneous contractile activity of coronary arteries is precipitated by increased cholesterol levels, but they may be a contributing or modulating factor, perhaps susceptible to calcium entry blockers.

c. PROSTAGLANDINS. Isolated human coronary arteries are contracted by products of cyclooxygenase activity such as prostaglandins F_{2alpha}, A_2, D_2, E_1, and I_2 (at concentrations greater than 1 μM) (382, 428, 617, 660, 661). PGF_{2alpha} was about equipotent with acetylcholine and norepinephrine (in the presence of propranolol) at contracting human coronary arteries, but the maximal contraction achieved by PGF_{2alpha} was about 75% greater than that elicited by either acetylcholine, serotonin, or depolarization and about 4-fold that stimulated by norepinephrine (413, 428). These PGF_{2alpha}-induced contractions were totally inhibited by 3 nM nifedipine.

Both spontaneous and prostaglandin E_2 (PGE_2)-induced contractions of human coronary arteries are depressed by arachidonic acid, and this depression could be reversed by cyclooxygenase inhibitors (660, 661). Human, bovine, and dog coronary arteries contract in the presence of aspirin, indomethacin, or ibuprofen (382, 593, 947), and acetylcholine-contracted sheep coronary artery strips were relaxed by arachidonic acid, the relaxation being reversed by indomethacin. Sheep coronary arteries contracted with acetylcholine also sometimes display rhythmic contractile activity similar to that seen in human coronary artery preparations, and this activity can be inhibited by indomethacin (762). Spontaneous rhythmic contractions of human coronary arteries in-

duced with PGF_{2alpha} (3 μM) were abolished by 0.5 μM diltiazem, but the increase in basal tension elicited by the prostaglandin was only inhibited by about 50% by 10 μM diltiazem (617). This tension was abolished by nifedipine (413).

Dog coronary artery preparations contracted after exposure to aspirin only if Ca^{2+} was present in the bathing solution. Diltiazem and nifedipine only inhibited these contractions by about 84 and 73%, respectively (947). The contractile effect of aspirin was reversible.

Contractions of cat coronary arteries induced by carbocyclic thromboxane A_2 (a stable analogue of thromboxane) were depressed by about 75% by nifedipine (IC_{50} about 0.14 μM). This effect of nifedipine was partially reversed by increasing the calcium concentration of the medium (997). A nifedipine-resistant component of some agonist-induced contractions has been observed by others (e.g., ref. 24), and there is some indication that this resistant component is also dependent on extracellular calcium (443, 445).

Prostacyclin is the major prostaglandin released spontaneously from rabbit, rat, and guinea pig hearts (198, 820, 1150) or released from human, dog, rat, and guinea pig hearts following injections of arachidonic acid (787, 833, 924, 973), and low concentrations (less than 1 μM) relax human coronary arteries (382). Alterations in the rate of production of prostacyclin, or another product of cyclooxygenase activity, might precipitate changes in tone of the coronary vasculature and perhaps also alter its reactivity to other contractile substances. These changes might be partially resistant to the effects of some calcium entry blockers.

d. NOREPINEPHRINE. In man, alpha-adrenergic-mediated vasoconstriction may contribute significantly to myocardial ischemia in patients with coronary artery disease (785), and norepinephrine contracts human and dog coronary arteries (166, 1095, 1107) but relaxes those of cat and sheep (107, 174, 175, 761, 762). Godfraind and Miller (428) could only elicit norepinephrine contractions in human coronary arteries in the presence of propranolol. The potency of norepinephrine in human coronary arteries was similar to that of acetylcholine and PGF_{2alpha}, but the maximal norepinephrine-induced contraction was only about one-half of that stimulated by depolarization, serotonin, or acetylcholine and about one-quarter of that stimulated by PGF_{2alpha}. It was concluded that alpha-adrenoceptors were probably not of great importance in young healthy coronary arteries, beta-adrenoceptor effects being predominant. However, it is known that beta-adrenoceptor-mediated relaxation of vascular tissue declines with age (336, 398), and while isoproterenol relaxed younger human coronary arteries contracted by depolarization, it had no effect on older arteries (428). Consequently, the contractile effects of norepinephrine may increase with age.

In dog coronary arteries, it has been shown that norepinephrine-induced contractions are almost completely abolished in calcium-free solutions or in the presence of 10 μM lanthanum. Also, norepinephrine only stimulated the efflux of prelabelled intracellularly stored calcium in the presence of external calcium (1095). Furthermore, these norepinephrine-induced contractions were completely inhibited by the calcium entry blockers lidoflazine (IC_{50} 2.3 μM), D 600, verapamil, and SKF 525A (24, 1095, 1107). Diltiazem and D 600 depressed contractions in human coronary arteries (382), and in dog, rabbit, pig, and human coronaries, about 20 to 40% of the norepinephrine-induced contraction was resistant to nifedipine (IC_{50} about 10 nM) (443, 445), even though this resistant component of contraction was also dependent on extracellular calcium. However, Angus and Brazenor (24) found that both norepinephrine and phenylephrine contractions of dog coronary artery could be abolished by nifedipine, but at very high (greater than 10 μM) concentrations. In this study, the optical isomers of verapamil were equiactive against alpha-adrenergic but not against serotonin- or K^+-induced contractions.

Thus, it would seem that contractions of coronary arteries stimulated by norepinephrine are totally dependent on extracellular calcium, norepinephrine being incapable of utilizing intracellularly stored calcium. In this respect, the dog coronary artery perhaps resembles the rat mesenteric artery (410) more than the rat or rabbit aorta (section II A 2).

Investigation of the contractile responses produced by various alpha-adrenoceptor agonists in blood vessels has suggested that those agonists such as clonidine and oxymetazoline, described as $alpha_2$-selective (665, 1019), induce responses which are dependent on extracellular calcium (431, 822). This may indicate that the alpha-adrenoceptors of the coronary circulation resemble $alpha_2$-adrenoceptors and that $alpha_1$-adrenoceptors are few in number or absent.

e. HISTAMINE. Histamine has been reported to produce contraction of larger human coronary arteries and relaxation of small arteries (654), or simply contraction (382, 383, 995, 996). Godfraind and Miller (427, 428) reported contraction, relaxation, or no effect in different human coronary arterial preparations, not seemingly related to vessel size, and also noted a decrease in sensitivity to the relaxant effects of histamine with increasing age (427). In animal tissues, the presence of H_1 and H_2 receptors in coronary vessels has been described, the different receptors subserving different functions (for example, 109, 381), and similar observations have been made in human coronary segments (382, 383). These differing observations could now probably be explained by a modulating effect of endothelium on histamine-induced contractile responses (1098).

In young human coronary arteries, histamine contractions were seen only at high concentrations. These contractions were not obviously concentration related and could still be elicited in calcium-free solution. Concentration-related contractions were, however, observed in

arterial preparations taken from a single 73-yr-old heart (Godfraind and Miller, unpublished observations). Ginsburg et al. (382) found that histamine contractions of human coronary arteries derived from heart transplant patients were noncompetitively antagonized by diltiazem or D 600. The reasons for these different sensitivities of histamine contractions to extracellular Ca^{2+} are unknown, but may be related to the age or pathological condition of the vessels. They do imply that calcium entry blockers are likely to have a greater antagonistic effect against histamine in older coronary arteries.

f. SEROTONIN. Human, pig, rabbit, sheep, and larger dog coronary arteries are contracted by serotonin (107, 166, 203, 428, 1049, 1107), but smaller arteries of the dog are not (880). In human arteries, serotonin was more potent than acetylcholine, norepinephrine, or PGF_{2alpha} (413, 428), and it was more potent than norepinephrine (variously about 2- or 100- to 300-fold) in larger dog arteries. In both human and dog vessels, the magnitude of the serotonin contraction was about 100% greater than that induced by norepinephrine. As in the case of histamine and acetylcholine, the variability in these observations must be regarded with caution due to possible uncontrolled influences of endothelium on the responses studied.

However, serotonin contractions of dog coronaries were not completely inhibited by lidoflazine (about 21% resistant), although the IC_{50} value was similar to that obtained against norepinephrine (1107).

Serotonin (10 μM) induced an increase in basal tension and the appearance of rhythmic contractions in some but not all human coronary arteries of various ages (428). In arteries obtained from very young hearts (9 and 15 yr old), serotonin induced contractions without any accompanying rhythmic activity, and these contractions were not inhibited by 1 or 10 nM nifedipine. In arteries from older hearts (about 45 yr old), serotonin induced an increase in basal tone and the appearance of rhythmic contractions in some preparations. Nifedipine abolished both of these effects but had a 10-fold greater potency against the rhythmic contractions with an IC_{50} of about 3.3 nM, while the IC_{50} value against basal tone was about 10 to 50 nM (428). Flunarizine also inhibited both tone and rhythmic activity induced by serotonin in arteries taken from a 45-yr-old heart, but had about equal potency in each instance (IC_{50} values of 72 and 83 nM, respectively). Rhythmic activity was not induced in arteries from a 43-yr-old heart, and the induced tone was less sensitive to the effects of flunarizine (IC_{50} 0.18 μM). These observations are also consistent with an increased sensitivity to the effects of calcium entry blockers with increasing age or changes in metabolic status (indicated by the presence of rhythmic activity), as have been described when contractions were induced by depolarization.

g. ERGONOVINE. Ergonovine has been proposed as a diagnostic agent for variant forms of angina (821, 971, 1129) as it provokes coronary arterial spasm in man (153, 1023) and contracts dog, rabbit, and human coronary arteries (508, 594, 613, 948, 1106). These contractions are inhibited by lidoflazine (IC_{50} about 1 to 5 μM) (1106). Atherosclerotic coronary arteries, and rat aorta without endothelium, are more sensitive to ergonovine (465, 508, 613), and it is likely that these ergonovine contractions are mediated mainly by stimulation of serotonergic receptors, at least in the dog (948).

h. ACETYLCHOLINE. Acetylcholine contracts human (428, 594, 996) and pig isolated coronary arteries, being about equipotent with histamine and more potent than epinephrine (995), without producing a change in membrane potential (560). These latter acetylcholine contractions are still present after prolonged exposure to calcium-free physiological solution. In guinea pig coronary artery, acetylcholine produces contractions associated with membrane hyperpolarization and was thought to increase membrane permeability to calcium and potassium and to liberate intracellularly stored calcium (638). These results imply that acetylcholine-induced coronary contractions should be at least partially resistant to the effects of calcium entry blockers, but direct information is lacking. The recent discovery of the powerful vasorelaxant effects of acetylcholine mediated by the endothelium in many arteries (356, 357) has complicated the interpretation of experimental results obtained when the status of the endothelium was unknown, but it has been reported that acetylcholine-mediated contractions of monkey, sheep, bovine, and pig coronaries are not affected by the presence of endothelium (166, 594).

i. ALKALOSIS. Alkalosis (pH change from 7.2 to 8.0) produces an increase in tone of rabbit coronary arteries. Increasing the external calcium concentration potentiated the contraction, and removing calcium abolished it as did diltiazem (IC_{50} about 50 to 60 nM) (1179).

j. ANOXIA. Induction of anoxia in dog coronary arteries already contracted by depolarization or serotonin elicited an increase in the established contractions that could be completely inhibited by lidoflazine (IC_{50} 1.2 and 6.5 μM, respectively) (1106). In the presence of norepinephrine-induced contractions, the increased contraction due to anoxia was also depressed by lidoflazine, the IC_{50} concentration being of similar magnitude (3 μM); however, in these circumstances, about 40% of the contraction was resistant to the effects of lidoflazine (1107).

k. CONCLUSION. The general potencies of calcium entry blockers in coronary arteries are comparable to those seen in large arteries and cerebral arteries. Coronary arteries are like cerebral arteries in that often a component of depolarization-induced contraction is resistant to inhibition by some, but not all, blockers.

5. Resistance vessels. In this section, the effects of calcium entry blockers in small peripheral resistance vessels are considered. In this context, the reactivity of vessels with an external diameter of less than 500 μm, usually about 100 to 400 μm, that are considered to

contribute to peripheral resistance in vivo, has been studied in vitro using a variety of methods, but notably that developed by Mulvany and Halpern (790, 791). Small cerebral arteries, already described in section II A 3, are of similar external diameter and have an equal claim to be considered resistance vessels.

a. ROLE OF MEMBRANE POTENTIAL. Norepinephrine (1 to 10 μM) stimulates a contractile response in branches of the mesenteric artery isolated from the rat. At lower concentrations of the agonist (1 to about 2.2 μM), this contraction (of up to 50% of the maximal response) was associated with a depolarization of the smooth muscle cell resting potential from about −59 mV to about −34 mV. Higher concentrations of norepinephrine did not produce further depolarization. An increase in K^+ concentration from 20 to 125 mM in the bathing solution produced a linear increase in the degree of membrane depolarization corresponding to 50 mV/decade. Tension increased over the range of 30 to 85 mM K^+ (792). In the potential range over which norepinephrine produced depolarization, K^+ only produced up to 38% of the maximal norepinephrine response, and the authors concluded that the mechanical response to norepinephrine may not generally be dependent on a depolarizing effect. In small rabbit mesenteric vessels, norepinephrine depolarization and contractile responses occurred over the same concentration range (0.1 to 100 μM), but as in the rat mesenteric vessels, the maximum depolarization was from about −60 mV to −30 mV (133). K^+ depolarization to the level of maximal norepinephrine-induced depolarization induced only about 25% of the maximal norepinephrine-induced contractile response. However, in vessels fully depolarized by K^+ (80 mM), norepinephrine stimulated ^{45}Ca entry to the same extent as it did in normal physiological solution. Membrane potential changes induced by norepinephrine in these small mesenteric vessels could then be secondary to Ca^{2+} entry via receptor-operated channels rather than due to a primary depolarization of the membrane and activation of potential-dependent Ca^{2+} channels. This interpretation is supported by the observation that, in the rabbit mesenteric resistance vessels, diltiazem abolished ^{45}Ca influx and the associated change in membrane potential (133).

b. ROLE OF EXTRACELLULAR Ca^{2+}: EFFECTS OF Ca^{2+} ENTRY BLOCKERS. After 10 min in Ca^{2+}-free (10 μM EGTA) physiological solution, maximal norepinephrine (10μM)-induced contractions of both rat and cat mesenteric resistance vessels were reduced by about 67%. The tonic component of the response (i.e., the stable contraction attained about 10 min after exposure to norepinephrine) was reduced by 96% in the rat, but by only 78% in the cat vessels. K^+ (124 mM) contractions were almost abolished by this treatment (993). In Ca^{2+}-containing solutions, nifedipine (0.3 μM) almost abolished K^+-induced contractions and (1 μM) reduced maximal (phasic) norepinephrine contractions by 25% in rat vessels and by 34% in cat vessels. Tonic norepinephrine contractions were reduced by 41 and 37% in rat and cat vessels, respectively. There was, therefore, a component of the norepinephrine contraction apparently dependent on extracellular Ca^{2+} that was not inhibited by nifedipine, and this component was larger in the rat vessels (993).

In rat vessels, Nyborg and Mulvany (837) found that removal of Ca^{2+} (5 mM EGTA) 15 s before depolarization (125 mM K^+) or application of norepinephrine (10 μM) almost abolished the tonic phase of contractile responses, reduced the phasic component of norepinephrine contractions by about 50%, and almost abolished those of K^+. They found that, while norepinephrine contractions (both phasic and tonic) were abolished by D 600 (0.1 mM), felodipine (0.1 μM) only reduced tonic contractions by about 55% and phasic contractions by about 25%. These concentrations of blockers are relatively high, and at 1 μM D 600, the degree of inhibition of both phases of contraction was similar to the maximal inhibition seen with felodipine and also broadly similar to the results of Skärby et al. (993) described above. Removal of Ca^{2+} from solutions bathing tissues contracted by K^+ or norepinephrine abolished the contractions (530). Nifedipine (30 nM) completely relaxed K^+-induced contractions (in the presence of 0.1 μM prazosin) but only relaxed established maximal norepinephrine contractions by about 25%. A 10-fold higher concentration of nifedipine had little further effect. However, both K^+- and norepinephrine-induced contractions were inhibited in an identical fashion by La^{3+}, and it was suggested that these results could be explained by two different pathways for Ca^{2+} entry in response to stimulation, one sensitive and one insensitive to the dihydropyridine, corresponding to potential-dependent and receptor-operated channels (530). While this might possibly be true in this vessel, contractile effects subsequent to receptor stimulation and Ca^{2+} influx are, however, antagonized by dihydropyridines in many other smooth muscles.

In isolated rabbit mesenteric resistance arterioles, norepinephrine contractions were almost entirely dependent on extracellular Ca^{2+} and were abolished by 3 μM diltiazem. Norepinephrine-stimulated ^{45}Ca influx and contractions were inhibited to about the same extent (82 to 92%) by 1 μM diltiazem; at this concentration of diltiazem, K^+ (80 mM)-stimulated ^{45}Ca influx and contractions were inhibited by about 60% (134). Taking into account IC_{50} values, diltiazem was about 55-fold more potent as an inhibitor of norepinephrine- than of K^+-induced contractions (table 3). Similar results were obtained in a perfused preparation of rabbit mesenteric resistance vessels (135).

Human mesenteric arterioles contracted by K^+ (127 mM) were relaxed almost completely (about 4% residual contraction) by $>$ 1 μM nifedipine or nimodipine, the respective IC_{50} values being 21 and 6.5 nM (102).

c. CONCLUSION. It is in the resistance vessels that substantial functional differences between receptor-operated and potential-operated calcium channels have

TABLE 3

IC_{50} values of calcium entry blockers on depolarization- and agonist-induced contractile responses and ^{45}Ca influx in resistance vessels

Blocker	Stimulant*	IC_{50} (nM)	Resistant to blocker (%)	Ref.
Rabbit mesenteric resistance vessels				
Diltiazem	Nor	30†		(136)
Diltiazem	K^+	600†		(136)
Diltiazem	Nor	10	0	(134)
Diltiazem	K^+	550	8	(134)
Rat mesenteric resistance vessels				
Nifedipine	Nor	71	59	(993)
Nifedipine	K^+	2	0	(837)
Nifedipine	Nor	15		(837)
D 600	K^+	4	0	(837)
D 600	Nor	288	0‡	(837)
Felodipine	K^+	0.005	0	(837)
Felodipine	Nor	4	50‡	(837)
Cat mesenteric resistance vessels				
Nifedipine	Nor	26	63	(993)
Human mesenteric resistance vessels				
Nifedipine	K^+	20	4	(102)
Nimodipine	K^+	6.5	4	(102)

* Abbreviation: Nor, norepinephrine.

† IC_{50} value on ^{45}Ca influx (all other IC_{50} values refer to contractile responses).

‡ Phasic and tonic responses were inhibited by about 25% and 55%, respectively, by felodipine but by 100% by D 600.

been demonstrated, even though differences are evident in all other vessels. The partial lack of antagonistic effect of dihydropyridines for agonist-induced contractions dependent on extracellular Ca^{2+} is probably not a general characteristic of receptor-operated channels, but rather of the population of receptor-operated channels being comprised of different proportions of channels resistant to dihydropyridines in different tissues (section III F). Generally, calcium entry blockers are more potent as antagonists of depolarization- than of agonist-induced Ca^{2+} influx and contraction, although confusion has arisen due to a component of agonist-induced contraction not dependent on extracellular Ca^{2+}. Diltiazem, however, is clearly more potent as an inhibitor of agonist- than of K^+-induced contraction and ^{45}Ca influx in rabbit resistance vessels, another indirect argument for differences between Ca^{2+} channels.

6. Veins. The hemodynamic effect of drugs is not only dependent on their action on heart and arteries, but also on their action on veins. Veins are a reservoir of variable capacity that can be actively modified when the smooth muscle cells in their walls contract or relax, in particular under the control of the sympathetic nervous system. In vitro studies have shown that some veins display rhythmic myogenic activity.

In dog saphenous veins, 50 μM diltiazem almost abolished K^+-induced contractions, and the IC_{50} value was about 1 μM (914). La^{3+} (5 mM) pretreatment abolished K^+-induced contractions but reduced maximal contractions evoked by a series of alpha$_1$-adrenoceptor agonists by variable amounts. For example, norepinephrine and phenylephrine contractions were inhibited by about 50% and methoxamine by about 70%. Nifedipine (1 μM) pretreatment did not significantly affect norepinephrine contractions but reduced those to phenylephrine and methoxamine by about 20 and 30%, respectively (575), although such differences between norepinephrine and phenylephrine have not always been seen (788). This concentration of nifedipine also failed to abolish ^{45}Ca uptake stimulated by these agonists (575). It was concluded that alpha$_1$-adrenoceptor agonists mobilize both intracellular and extracellular Ca^{2+} for contractions and that the sensitivity of ^{45}Ca uptake to inhibition by nifedipine was inversely related to the intrinsic ability of the alpha$_1$-agonists to translocate extracellular Ca^{2+}. High concentrations of verapamil (10 to 100 μM) have been found to displace norepinephrine concentration-effect curves evoked in dog saphenous veins to the right without affecting maximal responses (664), while diltiazem concentrations up to 100 μM had no effect at all (664, 914). Diltiazem (10 and 100 μM) did, however, shift phenylephrine concentration-effect curves (664) and inhibited (IC_{50} about 1 μM) acetylcholine-induced contractions (914). The maximal inhibition of acetylcholine contractions was about 40%.

The effects of verapamil and diltiazem as antagonists of norepinephrine- or phenylephrine-induced contractions these concentrations could perhaps be attributed to alpha-adrenoceptor blockade (see section III D). However, the differential effect of diltiazem, antagonizing phenylephrine but not norepinephrine contractions, is similar to the effect of nifedipine in the same vessel (575), and nifedipine has been reported not to interact with alpha-adrenoceptor binding sites (section III D). D 600 (50 μM) did not completely relax contractions of dog renal vein induced by either K^+ or norepinephrine (514).

Stretch- and temperature-induced tone increases in rabbit facial veins were inhibited in Ca^{2+}-free solutions but not by verapamil, nifedipine, or diltiazem (1164). In fact, high concentrations (10 μM) of the antagonists increased stretch-induced tone slightly, and 0.1 and 1 μM diltiazem increased temperature-induced contractile activity. Ca^{2+} concentration-effect curves in this tissue were antagonized by verapamil, nifedipine, and diltiazem, and pA_2 values of 7.5, 6.7, and 6.5 were calculated. These values for verapamil and diltiazem are comparable to values obtained in intestinal smooth muscle (table 5), but nifedipine is 2 to 3 orders of magnitude less potent. In arterial tissue also, nifedipine is active at concentrations one-tenth to one-hundredth of the active concentrations of verapamil and diltiazem (table 2).

Histamine-induced contractions of rabbit facial vein

were abolished in Ca^{2+}-free solution, but they were not affected by 1 μM verapamil, nifedipine, or diltiazem, although 10 μM verapamil and diltiazem shifted histamine concentration-effect curves to the right and depressed maximal responses (1164). This seems to be an effect of the blockers that is not due to an interaction with calcium channels.

Isolated rat, rabbit, and guinea pig portal veins exhibit spontaneous rhythmic activity, dependent on extracellular calcium (591, 1037). This activity is inhibited by a variety of calcium entry blockers over concentration ranges similar to those that antagonize K^+-induced contractions in other smooth muscles (444, 446, 763, 1105, 1108). According to Van Nueten and Vanhoutte (1108), the spontaneous activity of the rat portal vein can be inhibited by nifedipine and D 600, but not by flunarizine. Alpha-adrenoceptor agonists increase the frequency and amplitude of the venous spontaneous contractions and also increase the tone of the preparation, but only in the presence of Ca^{2+} (515). Norepinephrine-stimulated uptake of ^{45}Ca in rat portal veins was reduced by D 600 and nifedipine (1151), and norepinephrine contractions of this vein were antagonized by D 600 (0.1 to 1 μM) and the dihydropyridines nifedipine, nitrendipine, and nisoldipine (0.1 nM to 0.1 μM) (487, 1151). This antagonism by D 600 and nifedipine could be reversed by increasing the calcium concentration of the bathing solution (574). These concentration ranges are the same as those found to be active in arterial and intestinal smooth muscle. However, although in both rat and guinea pig portal veins the increase in amplitude of spontaneous activity induced by norepinephrine can be abolished by high concentrations (10 μM nifedipine, for example) of calcium entry blockers that abolish ^{45}Ca entry, the increased tone is only inhibited by 50 to 60% (446, 763).

A maximal inhibition of norepinephrine-induced contractions by 100 μM diltiazem has been reported in rat femoral vein (804). As in dog saphenous veins, a differential effect of diltiazem has been noted when contractions were induced by adding Ca^{2+} to rat portal veins prewashed in Ca^{2+}-free solution containing either phenylephrine or the $alpha_1$-adrenoceptor agonist UK 14304. When UK 14304 was the agonist, Ca^{2+} concentration-effect curves were displaced in a competitive fashion by diltiazem, but in the presence of phenylephrine, a non-competitive antagonism was evident (515). All these differential effects related to various alpha-adrenoceptor agonists could be explained by a relatively smaller ability of phenylephrine to facilitate the translocation of extracellular Ca^{2+} (431, 575). That could be related to a lower intrinsic efficacy of the agonists most susceptible to the blocker (section III F).

In isolated human pulmonary veins, nifedipine was about 10-fold more potent as an antagonist of K^+- than of norepinephrine-induced contractions (IC_{50} values about 30 nM and 0.20 μM, respectively), but the maximal inhibitory effect amounted to about 75% for K^+ and about 60% for norepinephrine with 25 μM nifedipine—a very large concentration (949). Similar observations were made with other human isolated veins (753–755). In man, nifedipine infused locally did not antagonize norepinephrine constriction of hand veins, but it did antagonize K^+-induced contractions and dilated forearm resistance vessels (920).

Overall, contractions in veins associated with membrane depolarization, whether spontaneous or K^+-induced, can be antagonized by calcium entry blockers over similar concentration ranges that have been found to inhibit contractions of this type in other smooth muscles. However, there are exceptions, such as rabbit facial vein, and 100% inhibition might not always be achieved. Agonist-induced contractions are often much less sensitive to the blockers than is usually the case in other vascular tissues. There may be many contributing factors implicated in this apparent lack of antagonism (section III F), but, considering the very high concentrations of blockers shown to be ineffective against contractions demonstrated to exhibit a dependence on extracellular Ca^{2+}, it might be questioned if some of these agonists do in fact activate a Ca^{2+} channel in veins (they certainly do not activate a potential-operated channel), or if they promote Ca^{2+} entry into veins predominantly by another mechanism.

B. Nonvascular Smooth Muscles

While the clinical uses of calcium entry blockers are, at the present time, directed towards cardiovascular problems, nonvascular smooth muscles have been used as models for the responsiveness of vascular smooth muscles and to elucidate possible mechanisms of action. Also, an understanding of the actions of compounds directed towards vascular smooth muscle in nonvascular types of smooth muscle might increase awareness of possible side effects of the drugs as well as point towards new therapeutic applications.

1. Intestinal smooth muscle. As in other varieties of smooth muscle, exposure to potassium-rich solutions elicits contractions that are dependent on extracellular Ca^{2+} (99, 422, 452, 453, 543, 1147). The kinetic features of Ca^{2+}-induced contractions (as distinct from contractions evoked by replacing normal physiological solution by one containing an increased concentration of K^+ and the normal concentration of Ca^{2+}) are not identical to those of vascular smooth muscle. In guinea pig ileum exposure to Ca^{2+} after a 10-min preincubation in Ca^{2+}-free, 127 mM K^+-containing solution results in a biphasic contraction, an initial phasic component that is followed by a slowly developing tonic contraction that also wanes over a prolonged time (90 to 120 min) to reach a low sustained level. Both these components are antagonized by verapamil (2 μM), La^{3+} (0.5 mM), and EGTA. A prolonged preincubation in Ca^{2+}-free, K^+-containing solution (60 min) abolished the second phase of contraction

(543). Low concentrations of La^{3+} (6 to 20 μM) selectively inhibited the phasic component of contraction.

Hurwitz et al. (543) concluded that activation of two types of calcium channel is associated with K^+-induced depolarization. Available comparisons of the potency of calcium entry blockers as antagonists of the phasic and tonic components of depolarization-induced contractions, which usually show a greater potency against tonic as compared to phasic contractions (table 4) would seem to support this idea, but other explanations are possible. For example, "use dependence" could be a factor, as has been demonstrated for nifedipine, diltiazem, and D 600 (88), or perhaps depolarization of the membrane alters the affinity of Ca^{2+} binding sites in the glycocalyx for Ca^{2+}, generating a momentary increase in the concentration of free Ca^{2+} close to the cell membrane and effectively increasing the Ca^{2+} gradient across it. This would thereby apparently decrease the effectiveness of calcium channel blockers.

Early observations suggested that acetylcholine-induced contractions were mainly caused by release of sequestered Ca^{2+} (235). Contractions can be elicited by acetylcholine after a prolonged period (60 min) in Ca^{2+}-free solution (543), indicating the presence of stocks of intracellular Ca^{2+}. Carbachol also releases intracellular Ca^{2+}, but this stock was quickly depleted (within 5 to 6 min) in the absence of extracellular Ca^{2+} (101). An earlier study using La^{3+} to inhibit Ca^{2+} entry found that both K^+- and acetylcholine-induced contractions were similarly dependent on extracellular calcium (453), and other muscarinic agonists have not been found to be dependent on extracellular Ca^{2+} to evoke contractions (1071). Some of these discrepancies may be explained by differing experimental conditions that alter the relative importance of Na^+-Ca^{2+} exchange as a mechanism of Ca^{2+} entry into intestinal smooth muscle subsequent to muscarinic receptor stimulation. But there is also evidence to show that carbachol is less dependent on extracellular calcium than is acetylcholine to evoke contractions in guinea pig *taenia coli* (769), perhaps due to differences in the interaction of these two agonists with muscarinic receptors. For the moment it must be concluded that experimental protocols to test the efficacity of calcium entry blockers in intestinal smooth muscle, as antagonists of contractile activity subsequent to muscarinic stimulation, should be designed to determine the relative importance of intracellular Ca^{2+} in the response. Norepinephrine contractions are reported to be mainly dependent on intracellular Ca^{2+} (213) and histamine contractions on extracellular Ca^{2+} (808).

Verapamil was about 10-fold (347) and (±)D-600 about 25-fold (925, 1071) more potent as an inhibitor of tonic than phasic components of K^+-induced contractions in guinea pig ileum (table 4), but the selectivity was restricted to the (−) isomer, which had a potency ratio of about 60, the (+) isomer inhibiting both phases equally (577). For nifedipine, the difference was about 10-fold,

TABLE 4

IC_{50} or RC_{50} values of calcium entry blockers in intestinal smooth muscle as antagonists of maximal (or near maximal) contractions induced by depolarization or various agonists*

Blocker	Stimulant	IC_{50} or RC_{50} (nM)		Ref.
		Phasic	Tonic	
Nifedipine	K^+		3.2	(626)
Nifedipine	K^+	35	3.4	(1071)
Nifedipine	CD	20	5	(1071)
Diltiazem	K^+		22	(626)
Diltiazem	K^+		52	(602)
Verapamil	K^+		79	(626)
Verapamil	K^+		18	(603)
Verapamil	K^+		60	(602)
Verapamil	K^+	500	40	(347)
Verapamil	Hist		150	(603)
D 600	K^+	800	30	(1071)
D 600	CD	1,000	20	(1071)
D 600	Ach		100	(769)
D 600	Carb		300	(769)
Lidoflazine	K^+		630	(626)
Bepridil	K^+	1,100	1,000	(347)
SKF 525A	K^+	18,000	20,000	(1071)
SKF 525A	CD	14,000	17,000	(1071)
Reserpine	K^+		1,000	(131)
Reserpine	Carb		1,000	(131)
Flurazepam	K^+	100,000	20,000	(553)
Diazepam	K^+	100,000	30,000	(553)
Cyproheptadine	K^+		1,000	(698)
La^{3+}	K^+		800	(453)
La^{3+}	Ach		800	(453)
Pimozide	Ach		250	(888)
Pimozide	Hist		220	(888)
Pimozide	Nor		3,500	(888)

* Abbreviations: IC_{50}, concentration producing 50% inhibition of contractile responses; RC_{50}, concentration producing 50% relaxation of contractions; CD, *cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide, a muscarinic agonist; Hist, histamine; Ach, acetylcholine; Carb, carbachol; Nor, norepinephrine.

but SKF 525A and its dichloro derivative (925, 1071) and bepridil (347) were about equiactive as antagonists of the two phases of contraction (table 4). Concentration-effect curves to Ca^{2+} in depolarized guinea pig ileum were antagonized noncompetitively by SKF 525A and its derivatives (1071). These calcium blockers also had a similar potency as inhibitors of both phasic and tonic components of contractions induced by the muscarinic agonist, *cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide, and in these experimental conditions the agonist contractions were dependent on extracellular Ca^{2+} (1071). Nifedipine and D 600 are more potent as

inhibitors of tonic than phasic components of acetylcholine- and carbachol-induced contractions (769). One μM verapamil and 5 μM diltiazem have been shown to abolish K^+-stimulated ^{45}Ca uptake in guinea pig ileum (602, 603).

The order of potency of calcium blockers as antagonists of K^+-induced contractions is nifedipine > verapamil ≥ diltiazem = flunarizine = cinnarizine > lidoflazine (table 4), and this same order is, not unexpectedly, that found from displacement of Ca^{2+} concentration-effect curves and calculations of "pA_2" values (table 5). It is difficult to justify quoting pA_2 values for calcium entry blockers, as a true competition with Ca^{2+} for a receptor site is improbable (a pseudo-competitive effect is gained by increasing the Ca^{2+} gradient across the membrane), and the slopes of Schild plots (30) are not always equal to 1, but it has been argued that they provide a comparative measure of potency of the compounds in a defined set of experimental conditions (1014), and this is undoubtedly true.

There is a group of compounds of various structures, not usually regarded as calcium entry blockers (or calcium antagonists), for which this type of activity has been shown in intestinal smooth muscle. In not all cases has direct evidence of inhibition of Ca^{2+} influx into smooth muscle been provided.

Reserpine has been demonstrated to have calcium entry blocking activity in this tissue (IC_{50} about 1 μM) against both K^+ (59 mM)- and carbachol (10 μM)-induced contractions (131). Reserpine is thus relatively more potent than SKF 525A. Some benzodiazepine derivatives have also been shown to exhibit calcium entry blocking-like activity. Diazepam and flurazepam both inhibit K^+-induced contractions and, like many other compounds, are more potent as inhibitors of the tonic than of the phasic component of contraction (553). Even though these compounds have a low potency as inhibitors of K^+-induced contractions (table 4), this is an interesting observation because it has been shown that a benzodiazepine receptor antagonist (PK 11195) (747) is able to interact with dihydropyridine binding sites in the heart (748). The opiate agonists, loperamide and fluperamide, have been shown to inhibit dihydropyridine binding and inhibit K^+-induced contractions in guinea pig ileum (911), and this could contribute to their efficacy as antidiarrheal agents. Pimozide, a specific dopamine antagonist in some (20) but not all situations (569), potently antagonized Ca^{2+}-induced contractions of guinea pig ileum in an apparently competitive manner with a pA_2 of 8.8, making it more potent than verapamil or diltiazem and comparable with nifedipine (table 5), and also antagonized acetylcholine- and histamine-induced contractions (888) with a potency similar to that of verapamil (table 4). Cyproheptadine, a potent serotonin and histamine antagonist, inhibited tonic K^+ contractions and associated ^{45}Ca uptake in guinea pig *taenia coli* (IC_{50} about 2 μM), and its potency was intermediate between those of D 600 and chlorpromazine (698). Cyproheptadine also preferentially inhibited the tonic phase of K^+-induced contractions and displayed use dependence.

TABLE 5
pA_2 values of calcium entry blockers in intestinal smooth muscle

Blocker	pA_2	Ref.
Nifedipine	9.5	(626)
Nifedipine	9.8	(1008)
Nifedipine	9.08	(551)
Diltiazem	7.65	(626)
Diltiazem	7.6	(1008)
Diltiazem	6.9	(551)
Verapamil	7.8	(626)
Verapamil	7.8	(1008)
Verapamil	7.36	(551)
Verapamil	7.85	(926)
(±)D 600	8.8	(577)
(−)D 600	8.9	(577)
(+)D 600	7.9	(577)
Lidoflazine	7.0	(626)
Flunarizine	7.6	(1008)
Cinnarizine	7.7	(1008)
Pimozide	8.8	(888)
Pimozide	7.5	(1008)
Loperamide	7.0	(911)

Binding experiments have demonstrated an allosteric interaction between diltiazem and dihydropyridines (see section III A 2), and diltiazem has been shown to potentiate the negative inotropic effects of nimodipine (207). Some attempts have been made to investigate the possible functional interactions between calcium blockers in intestinal smooth muscle of the guinea pig, but evidence of marked effects has not been found. Interactions between verapamil and nitrendipine (542) and nifedipine and D 600 (1183) were additive, and verapamil slightly attenuated nimodipine responses (1011), which would broadly indicate an interaction at a similar site. Spedding (1011) could detect no interaction between nimodipine and either nifedipine, diltiazem, or cinnarizine that could not be explained by a simple additive effect, but Yousif and Triggle (1183) using a different technique did demonstrate a small potentiation of nifedipine and D 600 inhibitory effects by diltiazem. These experiments demonstrate the known difficulties of interpreting functional effects in whole tissues in terms of complex receptor (i.e., calcium blocker binding site) interactions.

The tissue most widely used for the study of calcium entry blockers in intestinal smooth muscle has been the guinea pig ileum. This tissue usually serves as a model for smooth muscle in general, but there is no *a priori* reason to suppose that the physiology of all smooth muscles is identical, and, as discussed in section III F,

vascular smooth muscle exhibits pharmacological differences between tissues and species. It has been suggested that contractions of rat intestinal smooth muscle evoked by acetylcholine are relatively insensitive to the calcium entry blockers verapamil, nifedipine, and dazodipine (PY 108-068) compared to guinea pig tissue, even though preincubation of the rat tissue in calcium-free solution in undefined conditions abolished acetylcholine responses (49). However, these contractions were only partially (20 to 40%) inhibited by La^{3+}, and the difference could be simply one of the relative importance of intracellular calcium stores.

2. *Respiratory smooth muscle.* Studies on the actions of calcium entry blockers on respiratory smooth muscle were stimulated by clinical reports indicating that these drugs were effective in asthmatic patients. Therefore, we shall start this section by briefly reviewing these clinical data.

a. CLINICAL EXPERIENCE. Cinnarizine has been reported to decrease the incidence of asthma attacks (249). Nifedipine and verapamil antagonized the induction of asthma by exercise (45, 141, 176, 860, 862), without affecting bronchial tone in asthmatic patients (45, 860, 862), and abolished the rise in plasma histamine associated with exercise (45). Nifedipine has not always proven to be effective in all patients (858), however, and higher doses of verapamil might produce bronchoconstriction in some asthmatics (862). Nifedipine reduced allergic bronchoconstriction when administered before antigen challenge in one trial (498), but neither verapamil nor nifedipine was effective in others (861, 1001).

Bronchoconstriction induced by cold air inhalation was antagonized by nifedipine (497, 1003), but not by diltiazem (704). The reduction in peak expiratory flow produced by inhalation of histamine was antagonized by nifedipine and verapamil in some studies (45, 711, 879, 1159) but not in others (859, 862).

In patients with obstructive airways disease, verapamil produced no systematic change in airways resistance (915). This might be expected if there is no change in basal resistance in these patients or in asthmatics.

b. ANIMALS IN VIVO. In anesthetized guinea pigs, verapamil and nicardipine (1 mg/kg) reduced basal pulmonary airways resistance by about 30 and 50%, respectively, and partially inhibited the increased resistance induced by leukotriene D_4 (LTD_4), acetylcholine, and histamine (3–5). A lower dose of nifedipine (30 μg/kg) had no effect on basal resistance in guinea pigs, but antagonized histamine-induced bronchoconstriction in 3 of 5 animals tested (264). Histamine-induced bronchospasm in guinea pigs could also be inhibited by cinnarizine (2.5 mg/kg) if the cinnarizine was administered 90 min prior to the histamine (1104). Flunarizine was only effective at a 16-fold higher dose. This difference between cinnarizine and flunarizine is the opposite of that usually seen in vascular smooth muscle (see section II A) and lends support to the idea that the antagonistic effects of the compounds are not due to nonspecific effects, even though the active concentrations are much higher than those necessary to antagonize vascular smooth muscle contractile activity.

In anesthetized dogs, a dose of nifedipine that had potent effects on resting tracheal blood flow had only a small effect on resting tracheal smooth muscle tension (520). Nifedipine (200 μg/kg) completely inhibited PGF_{2alpha}-induced increase in resistance and reduced that of histamine by about 80% (712), and 12.5 mg of nifedipine reduced bronchoconstriction to inhaled methacholine and citric acid (118).

c. ANAPHYLAXIS. Anaphylactic shock induced in sensitized guinea pigs was inhibited by cinnarizine [50% effective dose (ED_{50}) 0.67 mg/kg] and flunarizine (ED_{50} 0.53 mg/kg) (35). In dogs, nifedipine (200 μg/kg) and verapamil (200 μg/kg) i.v. (713) and 12.5 mg of nifedipine administered as an aerosol (118) reduced the increase in pulmonary resistance induced by aerosol ascaris antigen. Verapamil has also been reported to be effective as an aerosol in dogs, but in this study nifedipine was ineffective (713). Verapamil (150 μg/kg) i.v. was also effective in the ascaris-sensitive model in sheep, although in this species, verapamil alone increased basal pulmonary resistance by about 240% and did not modify airway responses to histamine or carbachol inhalation challenge (940).

d. TRACHEAL PREPARATIONS-UNSENSITIZED. **i. Importance of extracellular Ca^{2+} for contraction.** Preparations of guinea pig trachea in Ca^{2+}-free physiological solution are contracted by K^+ to a smaller extent than in the presence of extracellular Ca^{2+} (36, 180). Maximal contractions induced by histamine were inhibited by about 60%, and those of PGF_{2alpha}, PGE_2, and the calcium ionophore calimycin (A 23187) were almost abolished, but maximal acetylcholine-induced contractions were not affected in Ca^{2+}-free physiological solution. However, submaximal contractions induced by acetylcholine and histamine were affected to a greater extent than the maximal contractions, and repeated contractions evoked by these latter two agonists were of diminishing magnitude. Exposure to Ca^{2+}-free EGTA-containing solutions (0.1 or 2.5 mM) abolished K^+-induced contractions and reduced histamine, PGF_{2alpha}, carbachol, and acetylcholine contractions to about 1% of control (36, 180). These observations and those of others (220, 1137) tend to suggest that contractions induced by A 23187, histamine, PGF_{2alpha}, PGE_2, and leukotriene D_4 in guinea pig trachea are entirely dependent on extracellular calcium. It is accepted that depolarization in solutions enriched in K^+ produces contractions due to the influx of extracellular calcium (see above).

The reported differences in sensitivity of contractile agonists to withdrawal of external calcium might mean that K^+, acetylcholine, and histamine are better able to mobilize any residual extracellular calcium than are the other agonists tested. In turn, this might mean that the

actions of acetylcholine and histamine, but not of the other agonists, are associated with varying degrees of depolarization of the smooth muscle cell membrane. Acetylcholine has been shown to depolarize dog trachealis muscle, there being a good correlation between depolarization and induced contraction for small concentrations of agonist (10 nM to 1 μM), but higher concentrations elicit greater contractions but no further depolarization (265), and in this tissue acetylcholine-induced contractions are also reduced in magnitude but not abolished in the absence of extracellular Ca^{2+} (266).

ii. Effects of calcium entry blockers. K^+-induced contractions of guinea pig trachea are completely inhibited by nifedipine, verapamil, diltiazem, nicardipine, bepridil, and PY 108-068 (4, 8, 1137), the IC_{50} values of diltiazem and nicardipine being comparable to values found in other types of smooth muscle (compare tables 2 and 6). The potency of nifedipine, on the other hand (table 6), was very much less than seen in vascular smooth muscle (table 2). Contractions elicited by Ba^{2+} in guinea pig trachea were abolished by 0.2 mM verapamil (123), and the IC_{50} value of verapamil for antagonism of Ca^{2+}, Ba^{2+}, and Sr^{2+} in this tissue is about 1.1 μM (36).

In general, the organic calcium antagonists are not very potent inhibitors of agonist-induced contractions of tracheal smooth muscle (table 6) in comparison with their potency in other types of smooth muscle (tables 2 and 4). However, it is interesting to note that there is some inhibitory activity in the case of a large number of antagonists and also that very large concentrations of some antagonists, such as bepridil, are inactive (4). Bepridil, as already mentioned, is a compound with a high lipid solubility which tends to concentrate in cells (179a, 783, 854a), and this lack of effect might be due to a rather short preincubation period (15 min) with the antagonist. Cinnarizine, another compound with a high lipid solubility, requires a prolonged pretreatment period (90 min) in vivo before an antibronchospasmodic activity is evident (1104).

Nitrendipine exhibits different antagonist properties in young and old guinea pig trachea. In young trachea, nitrendipine antagonism of Ba^{2+}-induced contractions was reversible, but in old trachea, the antagonism was irreversible (220).

Guinea pig tracheal preparations often develop spontaneous tone in normal physiological solution. This tone is abolished by Ca^{2+} removal but is not much affected by 10 μM verapamil (8, 220, 616). The resting tone (as distinct from spontaneous developed tone) of guinea pig trachea was reduced by 3 nM nifedipine, a concentration that shifted carbachol and histamine concentration-effect curves about 3- to 4-fold to the right. This shift was not increased in the presence of higher concentrations of nifedipine (264). In another study (152) 1 μM nifedipine reduced basal tone in guinea pig trachea, but did not antagonize concentration-effect curves elicited by histamine, methacholine, or serotonin. A 30-fold higher concentration of nifedipine shifted histamine concentration-effect curves to the right and inhibited maximal responses.

This type of antagonism, a shift of concentration-effect curves to the right, is not typical of a calcium entry blocker in other types of smooth muscle (see above), but it has also been noted in the case of antagonism of histamine-induced concentration-effect curves in guinea pig trachea by verapamil (10 μM to 1 mM) and in dog trachea where a high concentration of verapamil (0.1 mM), that abolished K^+-induced contractions, shifted acetylcholine-induced concentration-effect curves to the right. In this latter case, the displacement of the curves was not parallel, and lower concentrations of acetylcholine were inhibited while high concentrations were virtually unaffected (266). Nifedipine and verapamil also inhibited contractions induced by lower concentrations of carbachol in guinea pig trachea to a greater extent than higher concentrations, and 3 μM nifedipine did not completely inhibit contractions induced by any concentration of carbachol (9, 36). D 600, however, has been reported to produce some inhibition of maximal acetylcholine-induced contractions in dog trachea as well as a displacement of the concentration-effect curve at a concentration (50 μM) that inhibited K^+ contractions by about 90% (451). Not all studies have shown the same type of antagonism. Verapamil antagonized histamine contractions of guinea pig trachea noncompetitively (193) and was more potent as an antagonist of carbachol- than of histamine-induced contractions (123, 193) and least effective against PGF_{2alpha} (123).

In rat trachea methacholine-induced contractions were not affected by a 10-min preincubation with 1 μM nifedipine, although maximal K^+-induced contractions were inhibited by 10 nM nifedipine by about 80% and by more than 90% by 0.1 μM. Maximal calcium-induced contractions (in the presence of 60 mM K^+) were inhibited by about 90% by 10 nM nifedipine (152). However, nifedipine completely relaxed rat tracheal preparations precontracted with methacholine (10 μM). The IC_{50} value was 0.42 μM.

In guinea pig trachea, the nifedipine IC_{50} values for relaxation of contractions induced by 10, 30, and 100 μM histamine were 8.3 nM, 0.28 μM, and 0.63 μM, respectively (866), but preincubation with 1 μM nifedipine did not affect histamine concentration-effect curves (8, 866). Verapamil also relaxed established histamine contractions in guinea pig trachea but had about one-hundredth of the potency of nifedipine. This increased efficacy of nifedipine for relaxation of established contractions over inhibition by preincubation with the blocker was also seen with leukotriene-induced contractions in one study (866) but not in another (1137; see below) and with histamine-induced contractions in human tracheal strips in vitro (227). PGF_{2alpha}-induced concentration-effect

TABLE 6

−Log IC_{50} (M) values of calcium entry blockers on K^+ depolarization- and agonist-induced contractile responses in respiratory smooth muscle

Blocker	Agonist*	−Log IC_{50}	Inhibition (%)	Ref.
Guinea pig trachea				
Verapamil	Ach	4.75		(3)
Verapamil	LTD_4	4.1		(3)
Verapamil	Ach	4.17	100	(4)
Verapamil	Hist	4.73	100	(4)
Verapamil	5HT	5	100	(4)
Verapamil	K^+	6	100	(4)
Verapamil	Ach	4.1		(5)
Verapamil	Hist	4		(5)
Verapamil	Ach	4.17	100	(143)
Verapamil	Hist	4.73	100	(143)
Verapamil	LTD_4	4.55	100	(143)
Verapamil	5HT	5.74	100	(143)
Verapamil	K^+	6.05	100	(143)
Verapamil	K^+	6		(1137)
Verapamil	Meth	3.70		(123)
Verapamil	Hist	4.56†	100	(152)
Verapamil	Hist	5	100	(193)
Verapamil	Carb	5	100	(193)
Verapamil	Ca^{2+}	1.1	100	(36)
Verapamil	Ba^{2+}	1.1	100	(36)
Verapamil	Sr^{2+}	1.1	100	(36)
Diltiazem	Ach	3.91	100	(4)
Diltiazem	Hist	3.91	100	(4)
Diltiazem	SHT	4.02	100	(4)
Diltiazem	K^+	6.94	100	(4)
Diltiazem	Ach	3.91	100	(143)
Diltiazem	Hist	3.91	100	(143)
Diltiazem	5HT	4.02	10	(143)
Diltiazem	K^+	6.34	100	(143)
Nicardipine	Ach	4.76		(3)
Nicardipine	LTD_4	4.65		(3)
Nicardipine	Ach	4.53	100	(4)
Nicardipine	Hist	4.69	100	(4)
Nicardipine	5HT	5.02	100	(4)
Nicardipine	K^+	7.99	100	(4)
Nicardipine	Ach	4.5		(5)
Nicardipine	Hist	4.5		(5)
Nicardipine	Ach	4.53	100	(143)
Nicardipine	Hist	4.69	100	(143)
Nicardipine	LTD_4	<4	100	(143)
Nicardipine	5HT	5.02	100	(143)
Nicardipine	K^+	7.99	100	(143)
PY 108-068	Ach	3.45		(4)
PY 108-068	Hist	4.50		(4)
PY 108-068	5HT	4.15		(4)
PY 108-068	K^+	6.5		(4)
Bepridil	Ach	<4		(4)
Bepridil	Hist	<4		(4)
Bepridil	5HT	<4		(4)
Bepridil	K^+	4		(4)
Nitrendipine	LTC_4	<<4		(941)
Nitrendipine	AA	5.3		(941)
Nifedipine	K^+	6		(1137)
Nifedipine	Hist	6.55†	100	(152)
Etafenone	Ach	6.85		(639)
Etafenone	5HT	5.85		(639)
Etafenone	$PGF_{2\alpha}$	6.85		(639)
Rabbit trachea				
D 600	K^+	6.3		(841)
Rat trachea				
Nifedipine	Meth	6.38		(152)
Nifedipine	K^+	>8		(152)
Human trachea				
Nifedipine	Hist	5.5		(227)
Guinea pig trachea (sensitized)				
Verapamil	Antigen	5.73		(1144)
Verapamil	Antigen	3.66		(1145)
Verapamil	Hist	3.7		(1145)
Verapamil	Ach	3.7		(1145)
Nifedipine	Antigen	6.13		(1144)
Guinea pig parenchyma (non-sensitized)				
Verapamil	Hist	5.3	100	(193)
Verapamil	Carb	5.7	100	(193)

* Abbreviations: Ach, acetylcholine; LTD_4, leukotriene D_4; Hist, histamine; 5HT, serotonin; Meth, methacholine; Carb, carbachol; LTC_4, leukotriene C_4; AA, arachidonic acid; $PGF_{2\alpha}$, prostaglandin F_{2alpha}.

† These values were determined from experimental protocols involving the relaxation of precontracted preparations. All other values were determined using experimental protocols involving preincubation with the blocker and a subsequent challenge with contractile agonist.

curves were unaffected by 10 μM verapamil, although carbachol concentration-effect curves were antagonized (36).

LTD_4 (50 nM) did not stimulate ^{45}Ca uptake into guinea pig tracheal smooth muscle, although it evoked a contraction that was antagonized by about 35% by 1 μM verapamil. K^+ (90 mM) induced contractions and ^{45}Ca uptake, but while verapamil inhibited the contractions by about 35%, it abolished the ^{45}Ca uptake (890). In another study (1137), LTD_4-induced contractions were abolished in the absence of external calcium (0.1 mM EGTA). Verapamil and nifedipine (1μM) antagonized LTD_4 contractions by about 20 to 30% and K^+ (100 mM) contractions by about 45 and 75%, respectively. Diltiazem (1 μM) did not inhibit either contraction. Nifedipine, verapamil, and diltiazem (0.1 mM) relaxed trachea precontracted with LTD_4 by only about 37, 19, and 15%, respectively. These results are difficult to reconcile. Some of the differences might be explained by peculiarities in calcium mobilization and handling in this tissue.

Overall, calcium entry blockers show some degree of inhibitory actions in trachea, but they are generally less potent than in other smooth muscles from the vascular system and intestine.

e. PARENCHYMA. Responses to LTD_4 were inhibited in parenchyma in Ca^{2+}-free solution containing La^{3+}, whereas in trachea, omission of calcium or addition of lanthanum alone was sufficient to produce inhibition (941). LTD_4-induced contractions of guinea pig parenchyma were inhibited but not abolished in Ca^{2+}-free solution as they were in trachea (1137). In these conditions, LTD_4 probably initiated contraction indirectly via thromboxane, because it was blocked by meclofenamic acid (1136). Nifedipine and verapamil were more effective antagonists of histamine-induced contractions in guinea pig parenchyma than in trachea of the same animals (193, 263), but effective concentrations of verapamil were in the range of 1 μM to 1 mM. Similarly, verapamil was about 5-fold more potent as an inhibitor of carbachol-induced contractile responses in parenchyma than trachea (193). However, this apparent selectivity of calcium entry blockers for parenchyma over tracheal smooth muscle is not true of all agonists; LTD_4 concentration-effect curves were antagonized to a greater extent by nifedipine (1 μM) in trachea than in parenchyma (1137). Nifedipine (3 nM to 3 μM) exhibited a greater antagonism for carbachol than histamine in one study, shifting carbachol concentration-effect curves to the right to a greater extent (about 5- to 50-fold) than were concentration-effect curves to histamine (shifted about 2- to 5-fold) (264). But in another report, concentration-effect curves to both histamine and carbachol were shifted by about 3- to 4-fold to the right by 3 nM nifedipine, and larger concentrations produced no greater shift.

f. SENSITIZED TRACHEA. Contractions induced in sensitized tissues by exposure to the sensitizing agent (the antigen) are of course due to the liberation of active products from tissue components, such as mast cells. This secretory process is dependent on extracellular Ca^{2+} (see section II F). Therefore, any interpretation of the effects of calcium entry blockers on antigen-induced contractions must account for the possible effects of the drug on the secretory process, as well as for its direct effects on the action of the liberated active products at the smooth muscle cells.

Antigen-induced contractions of guinea pig trachea are reported to be more dependent on intracellular Ca^{2+} than are contractions induced by other means in unsensitized tissue. La^{3+} (1 mM) had no effect on ovalbumin-induced contractions and, in fact, enhanced contractions induced by the ionophore A 23187 (119). The slow reacting substance of anaphylaxis (SRS) has also been shown to release ^{45}Ca from microsomes prepared from sensitized but not from desensitized guinea pig lungs, which is consistent with an increased independence of extracellular calcium for antigen-induced contractions (493).

Verapamil (100 μM) and nitrendipine (280 μM) abolished the ovalbumin-induced contractions in guinea pig trachea, 21 μM flunarizine had no effect, but 280 μM produced an inhibition of about 60% (119, 1145). These concentrations of calcium entry blockers, while high, are not much different from those reported to be effective in unsensitized tissue (table 6), and histamine-induced contractions of sensitized and unsensitized guinea pig trachea are equally sensitive to inhibition by verapamil (723).

g. SENSITIZED PARENCHYMA. Antigen-induced responses were more dependent on extracellular calcium in parenchyma than in trachea of the same guinea pigs as determined in calcium-free solution. Both nitrendipine and flunarizine were less effective as inhibitors of antigen-induced contractions in parenchyma than in trachea (119).

h. CONCLUSION. The various observations reported are conflicting, reflecting the complex nature of induced bronchoconstriction, not least the possible involvement of a mixture of contractile agents with differing susceptibilities to calcium entry blockers in respiratory tissues. The mixture of contractile agents may also vary with the stimulus provoking the bronchoconstrictor response. Nevertheless, some encouraging results with calcium entry blockers have been reported in clinical situations.

3. Vas deferens. Potassium causes a biphasic contraction of vas deferens of the rat. An initial rapid (phasic) contraction that peaks in about 20 s is followed by a relaxation to a sustained (tonic) level of contraction (1041). Part of the phasic response can be attributed to the liberation of norepinephrine from the abundant sympathetic nerve terminals in this tissue (1004), and removal of external Ca^{2+} antagonizes, but does not completely abolish, depolarization-induced contractions (490). The separated prostatic and epididymal portions display a similar behavior, except that the time to peak of the phasic contraction is reduced to about 10 s in the prostatic portion, and the following relaxation is more rapid and profound before the contraction recovers to a stable plateau. Also, the contribution of norepinephrine to the phasic response of the prostatic portion is very much less than in the epididymal portion (490).

The residual K^+ contraction of whole rat vas deferens in the absence of external Ca^{2+} was almost abolished by 0.29 μM nifedipine. Verapamil and D 600 abolished K^+-induced contractions, but about 15 to 20% of both phasic and tonic components was resistant to blockade by nifedipine. Ca^{2+} concentration-effect curves (128 mM K^+) were noncompetitively inhibited by nifedipine, verapamil, and D 600 (490), and SKF 525A (1071).

Flunarizine, verapamil, and D 600 exhibited a selectivity for the tonic component of contraction (490, 1071). Nifedipine showed a selectivity for the tonic component in one study (1071), but not in another (490). This selectivity is probably the manifestation of the "use-dependent" effect of these blockers.

Nifedipine antagonized contractions evoked by hypergastric nerve stimulation in rat and guinea pig vas def-

erens and the associated action potentials (76, 1036). Ca^{2+} is the principal current-carrying ion in the action potential response (65).

A short incubation with SKF 525A (30 μM for 5 min) inhibited maximal K^+ (100 mM)-induced contractions by about 80% without significantly affecting norepinephrine-induced responses in rat vas deferens, and Ca^{2+} concentration-effect curves elicited in the presence of 60 mM K^+, but not in the presence of norepinephrine (0.1 mM), were inhibited noncompetitively by SKF 525A (1040). These observations could be interpreted to mean that norepinephrine does not activate potential-dependent Ca^{2+} channels in this tissue and that SKF 525A does not interfere with intracellular calcium mechanisms associated with contraction. This would mean that the entry of calcium stimulated by norepinephrine must be by another mechanism, either a distinct receptor-operated channel or another means, such as Na^+-Ca^{2+} exchange. However, it was shown in a later study (1071) that a 30-min preincubation with SKF 525A (10 to 100 μM) inhibited both K^+- and norepinephrine-induced contractile responses with similar IC_{50} values (table 7). In this study, D 600 was also equipotent against norepinephrine and K^+ contractions, nifedipine was more potent as an antagonist of K^+ than norepinephrine, but the dichloro derivative of SKF 525A was more potent against norepinephrine- than K^+-stimulated responses (table 7).

Verapamil (20 μM) relaxed vas deferens of the rat precontracted by KCl (124 mM) or phenylephrine (50 μM), and pretreatment with 50 μM verapamil for 30 s practically abolished subsequent phenylephrine responses (215). Evidently, the calcium entry blockers that have been studied are much less potent in vas deferens than in vascular smooth muscle.

4. Uterus. As first shown by Fleckenstein and Grün (315), calcium entry blockers (verapamil, D 600, and prenylamine) suppress excitability (spontaneous Ca^{2+}-mediated spike potentials) and contractility in rat uterus. Further studies also showed that these drugs abolish spike discharge evoked by electrical and pharmacological stimuli and the concomitant phasic contractions (reviewed in ref. 303).

The sensitivity of uterine contractions to calcium entry blockers may vary according to the stimulus. K^+-induced contractions of rat uterine muscle were abolished by 1.5 mM La^{3+}, a concentration that reduced acetylcholine contractions by about 80% (453). K^+ contractions were about 100-fold more sensitive to La^{3+} inhibition than were acetylcholine contractions. The potencies of several calcium entry blockers against spontaneous and oxytocin-induced tension development by the uterus from the term pregnant rat were determined by Granger et al. (460). The rank order of potency was nifedipine > D 600 > diltiazem > cinnarizine. Spontaneous contractions were more sensitive to these drugs (IC_{50}, 0.44, 6, 6.6, and 1300 nM, respectively) than were oxytocin-induced contractions (IC_{50} 7.2, 11.5, 1700, and 7900 nM, respectively). Cinnarizine was thus much less potent than the other drugs on the uterus, whereas it was the second most potent compound against KCl-induced pressure rises in the mesenteric bed.

K^+ (135 mM)-stimulated ^{45}Ca influx into rat uterine cells was abolished by nitrendipine (10 nM) and D 600 (2 to 10 μM) and also inhibited by high concentrations (IC_{50} 4 μM) of diethylstilboestrol (50, 658, 768, 1073). Ca^{2+} concentration-effect curves were displaced to the right by PN 200–110, nitrendipine, nifedipine, D 600, and diltiazem, and respective calculated pA_2 values were 10.63, 9.56, 9.41, 9.05, and 7.57 (469). A reasonably good correlation for these pA_2 values for PN 200–110, nitrendipine, and nifedipine and pK_i values determined by displacement of [^{3}H]nitrendipine binding was obtained, but not for diltiazem which potentiated dihydropyridine binding, or for D 600 which had a much lower K_i than pA_2 value (469). These discrepancies can be explained by differences and interactions between calcium entry blocker binding sites (section III A).

TABLE 7

IC_{50} values of calcium entry blockers in rat vas deferens

Blocker	Stimulant*	IC_{50} (μM)		Resistant (%)	Ref.
		Phasic	Tonic		
Verapamil	K^+	1.85	0.94	0	(490)
Nifedipine	K^+	0.09	0.05	15–20	(490)
Nifedipine	K^+	0.06	0.01		(1071)
Nifedipine	Nor	0.2	0.9		(1071)
D 600	K^+	1.99	0.88	0	(490)
D 600	K^+	3	0.28		(1071)
D 600	Nor	3	0.8		(1071)
Flunarizine	K^+	27.9	2.79	0–10	(490)
SKF 525A	K^+	15	12		(1071)
SKF 525A	Nor	35	28		(1071)
2Cl-SKF 525A	K^+	11.5	16		(1071)
2Cl-SKF 525A	Nor	9	8		(1071)

* Abbreviation: Nor, norepinephrine.

C. Cardiac Tissues

In this section, we shall consider first ionic currents in cardiac cells, and how they are modified by calcium entry blockers. Then, the functional effects of these drugs on pace-maker activity, conduction, and contractility will be briefly considered [this topic has been extensively reviewed by Singh et al. (992) and by Fleckenstein (303)]. Thereafter, their actions in various experimental pathophysiological models will be examined.

1. Ionic currents. The electrical activity of the heart is the result of different ionic membrane currents that determine the shape of the observed cardiac action potentials. The main evidence for the existence of various

inward currents has been obtained in voltage-clamp experiments measuring ionic currents during voltage steps from −80 mV to 0 mV. The major current components of an action potential of a ventricular muscle fiber consist of a rapidly activating and inactivating Na^+ inward current (I_{Na}), a more slowly inactivating and activating secondary inward current, predominantly carried by Ca^{2+} (I_{si} or I_{Ca}), and also outward currents both time independent and time dependent that are mainly carried by K^+. It has been proposed that these ionic currents flow through channels opened by depolarization. The membrane events have been interpreted according to the gating mechanism first proposed by Hodgkin and Huxley (524). According to this hypothesis, used to describe the sodium-dependent action potential, depolarization to a threshold voltage results in a very rapid opening of the activation (m) gates of sodium channels. If the inactivation (h) gates of these channels have not already closed, the channels are now open or activated, resulting in a large increase in sodium permeability. Sodium then diffuses down its electrochemical gradient (from the extracellular towards the intracellular side of the membrane), and the membrane potential approaches the sodium equilibrium potential. This large sodium current is very brief, because depolarization, while opening the fast moving m gates, also causes inactivation of sodium channels by closure of the slower h gates. These sodium channels have been termed the fast channels. The depolarization caused by the sodium current produces phase 0 of the action potential and leads to activation of calcium channels and, more slowly, of potassium channels. Calcium channels become activated and inactivated in the same way as sodium channels, but in the case of calcium, the transitions occur at more positive potentials and more slowly; therefore, calcium channels have been termed "slow channels" (904, 931). The plateau of the action potential (phases 1 and 2) reflects the turning off of sodium current, the evolution of calcium current, and the slow appearance of a repolarizing potassium current. Final repolarization (phase 3) of the action potential results from eventual inactivation of calcium channels and an increase in potassium permeability in such a way that the membrane potential approaches the potassium equilibrium potential.

The slow inward current in cardiac cells has recently been reexamined (829). Work with single cells has shown that the current associated with voltage-dependent calcium channels activates and inactivates more rapidly than previously thought, and is, in fact, a fast current ($I_{Ca,f}$). According to Noble (829), I_{si} might be subdivided as follows:

$$I_{si} = I_{Ca,f} + I_{Na,Ca} + I_{Ca,s}$$

$I_{Na,Ca}$ is carried by the electrogenic Na^+-Ca^{2+} exchanger and reflects the entry of 3 Na^+ in exchange for the extrusion of 1 Ca^{2+}. $I_{Ca,s}$ is a very slowly inactivated calcium current that is not blocked by Ca^{2+}. As this terminology has not yet been used in studies dealing with calcium channel blockers, we shall continue to refer to I_{Ca} or I_{si} in the following discussion.

Subclassification of cardiac tissue components is based on action potential determinants (370, 992). In fast channel tissues (the His-Purkinje system, and atrial and ventricular muscles) the action potential is mainly dependent upon inward sodium current. In slow channel tissues (sinoatrial and atrioventricular nodes) the charge carrier is largely calcium (see fig. 5).

As shown by Fleckenstein (295, 303), calcium entry blockers produce excitation-contraction uncoupling in myocardium (fast channel tissue), i.e., they are able to markedly reduce the contractile response of the myocardial tissue while producing only limited changes in the action potential. The upstroke velocity and height of overshoot remain unaffected. However, the plateau phase is abbreviated, since Ca^{2+} influx cannot contribute to the maintenance of depolarization during this phase in the presence of calcium entry blockers. Another important feature of the inhibitory action of phenylalkylamines is its use dependence, which will be discussed in section III B 2. In contrast with specific calcium entry blockers, perhexiline and fendiline, for example, are less selective inhibitors of Ca^{2+}-mediated events and depress Na^+-dependent action potential parameters at concentrations that inhibit tension development by more than 50 to 70%.

Metabolic and ionic manipulations can alter the configuration of action potentials. Fast sodium currents are inactivated when cardiac muscles are immersed in physiological solution containing 20 to 25 mM KCl, and the addition of catecholamines allows slow potentials carried by calcium ions to be produced. These slow potentials are inhibited by calcium entry blockers (fig. 6) (303, 646, 667, 1017), and the effects on slow potentials can be correlated with the effects on tension development (303). The action of catecholamines on slow channels appears to result from a cyclic AMP-dependent phosphorylation of the channels (906), an indication of the dependence of the channels on cellular metabolism.

In view of the complexity of the underlying ionic events, interpretation of drug actions based on analysis of action potentials is difficult. Indeed, inhibition of slow action potentials may be due to a decrease in calcium conductance, but could also be the result of a change in potassium conductance. More definitive conclusions can be drawn from voltage-clamp experiments, which allow selective measurement of transsarcolemmal Ca^{2+} currents. Fleckenstein and coworkers (645, 646) showed that verapamil and D 600 suppressed I_{Ca} and contractility over the same concentration range, while leaving I_{Na} unaffected, and that their inhibitory effects could be overcome by increasing the external Ca^{2+} concentration.

In spite of their selectivity as calcium entry blockers,

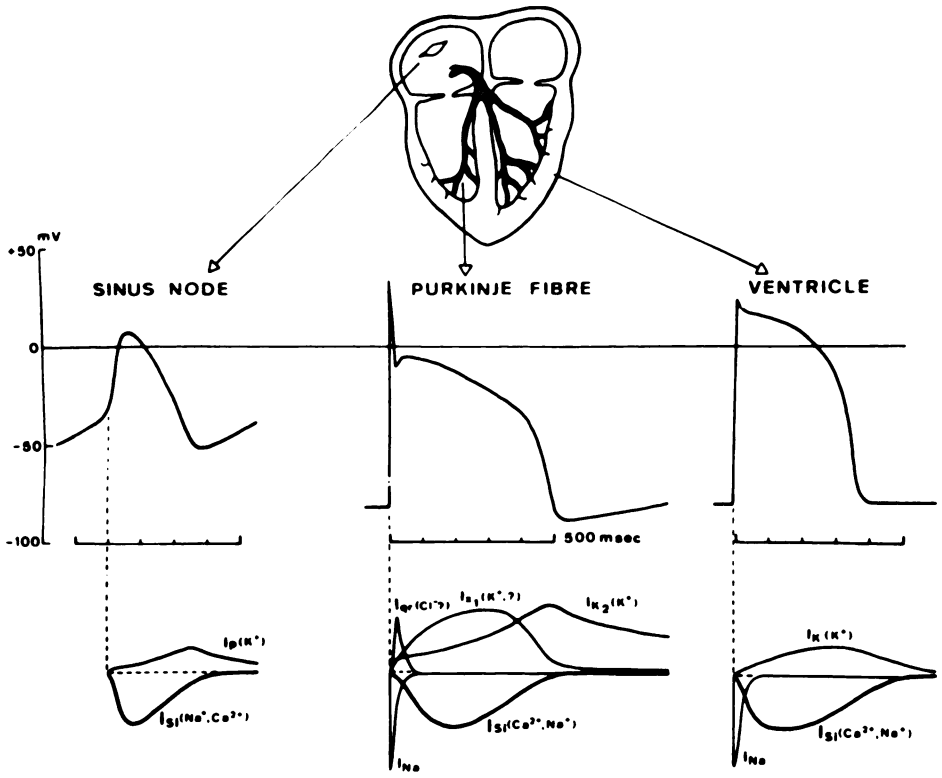

FIG. 5. Action potentials and underlying ionic currents in various cardiac tissues. I_{si} consists probably of several components (see text). Not detailed in the text: I_p is an outward potassium current, the deactivation processes of which are responsible for the pace-maker activity in nodal cells; I_{K2} is a similar potassium current in Purkinje fibers; I_{x1} and I_K are outward repolarizing currents in Purkinje and ventricular cells, respectively; I_{qr} is a special early repolarizing current in Purkinje fibers. The action potential is the resultant of the various inward and outward currents. Adapted from Gargouil (370).

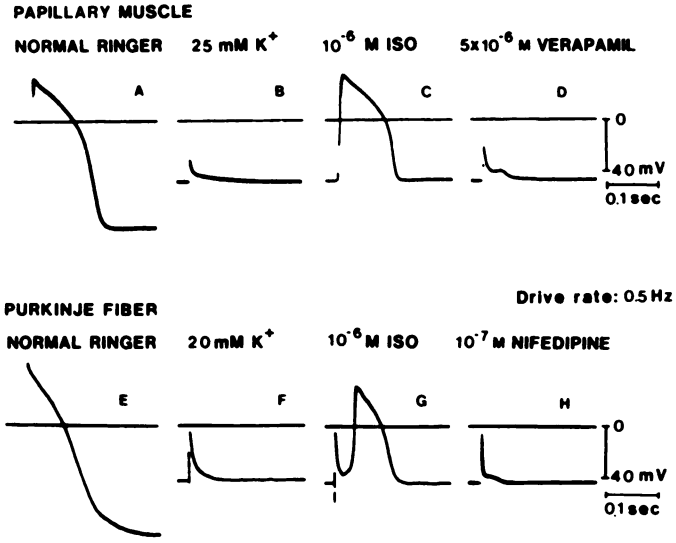

FIG. 6. Effect of verapamil and nifedipine on slow action potentials in guinea pig papillary muscle (*top*) and Purkinje fiber (*bottom*). Normal fast action potentials are shown (*A* and *E*). Elevation of extracellular K^+ to 20 to 25 mM depolarized to −45 mV and blocked excitability (*B* and *F*). Addition of isoproterenol (*ISO*, 1 μM) rapidly induced slow action potentials (*C* and *G*). Addition of verapamil (5 μM) or nifedipine (0.1 μM) blocked the slow action potentials (*D* and *H*). The *straight line* in each panel represents the zero potential level. Adapted from Sperelakis (1017).

verapamil and D 600 have multiple electrophysiological effects at high concentrations (53, 241, 894). Bayer et al. (53) have shown that they inhibit fast inward current, an indication that at higher concentrations they interact with Na^+ channels, and this effect also probably explains their local anesthetic effect (923). This action on Na^+ channels is more pronounced with the (+) isomers of verapamil and D 600 (see section III B 1). Galper and Catterall (364) have shown that inhibition of fast sodium channels in cultured cardiac cells by D 600 was antagonized by Ca^{2+}. The K_i for D 600 in the presence of 1.8 mM Ca^{2+} was approximately 3 μM. Studies by Kass and Tsien (610), Siegelbaum and Tsien (988), and Siegelbaum et al. (989) in Purkinje fibers have shown that D 600 inhibits not only I_{si} but also outward K^+ currents. Chen and Gettes (150) have studied the action of verapamil in guinea pig and canine papillary muscle and concluded that verapamil affects the slow outward as well as the slow inward current.

Dihydropyridines appear to be more specific than phenylalkylamines for the slow current I_{si}. For instance, Bayer et al. (55) have reported that nifedipine does not affect fast Na^+ inward current. Kass (609) has shown that nisoldipine inhibits I_{si} at very low concentrations (50% inhibition at about 0.02 μM) and, when tested at

concentrations as high as 10 μM, has no influence on the outward potassium currents (I_K). More recently, Hume (541) has studied the action of three chemically different calcium entry blockers (D 600, diltiazem, and nisoldipine) on Ca^{2+} and K^+ channels in single frog atrial cells in which time-dependent I_K is not a Ca^{2+}-activated K^+ conductance mechanism. He noticed that the extent to which the blockers discriminate between I_{Ca} and I_K is highly dependent upon the experimental or physiological conditions. Indeed, the block of time-dependent K^+ current was more effective at negative membrane potentials and, at low physiological rates of stimulation, I_K was more affected than I_{Ca}, the reverse being observed at higher rates. Nisoldipine was more selective for I_{Ca} than were the other compounds, as was observed by Kass (609).

Bepridil, an antianginal drug, decreases the slow inward current in cardiac tissue, but this drug is not very selective for the calcium channel, since it exerts an inhibitory action on the fast sodium channel over a similar concentration range (25, 663). Its electrophysiological effects resemble those of both class Ib and class IV antiarrhythmic drugs (25). Bepridil also depressed calcium spikes induced by tetraethylammonium in rat aortic cells (781).

Recently, it has been shown that some dihydropyridine derivatives, e.g., Bay K 8644 (967–969) and CGP 28392 (696, 1075), increase Ca^{2+} influx through voltage-dependent Ca^{2+} channels in heart and smooth muscle tissue (see also section III B).

2. Pace-makers, conduction, and contractility. a. PACEMAKERS AND CONDUCTION. Experimental data summarized above show that the effects of calcium entry blockers in cardiac tissues may vary according to several factors including the agent examined, the conditions of stimulation, and the degree of Na^+ and Ca^{2+} dependency of the action potential of the cardiac tissue under study. The electrophysiological effects of calcium entry blockers are primarily observed at the atrioventricular (AV) and at the sinoatrial nodes. In isolated preparations, sinus node frequency is markedly slowed due to depression of the rate of spontaneous diastolic depolarization, but there is no effect on threshold potential (294, 302, 323). This reduction is concentration dependent. In a comparative study on rabbit heart, Kawai et al. (615) found that diltiazem, verapamil, and nifedipine produced a similar slowing of the spontaneous beat over the concentration range 0.1 to 1 μM. Similar concentration-dependent effects were found in the isolated AV node, especially in the upper and middle portions in which the slow-responding fibers were depressed while the resting membrane potential was not altered. Diltiazem, verapamil, and nifedipine prolong refractory periods in the excised rabbit AV node. Motomura and Taira (779) have shown that enhanced automaticity evoked by norepinephrine perfusion of AV node preparations was reduced by calcium entry blockers, when injected into the posterior septal artery which supplies the proximal portion of the AV junctional area; verapamil, in these conditions, was more potent than nifedipine. Bepridil (5 to 50 μM) reduces spike frequency in isolated sinoatrial tissue (58, 614).

In intact animals and in clinical conditions, the most measurable effects are on the AV node, and most observations have been made on the effects of verapamil. In anesthetized dogs, there is a relation between plasma levels and electrophysiological effects. For instance, when verapamil plasma levels were <150 ng/ml, PR was lengthened from 392 to 442 ms, and this increased to 554 ms at plasma levels between 150 and 400 ng/ml; atrial-His conduction times. (AH intervals) were, respectively, equal to 65, 94, and 136 ms (714). Nisoldipine and diltiazem also lengthened intervals in open chest dogs (667). In patients, it has been observed that diltiazem and verapamil reduce AV conduction, but that nifedipine, in clinically practical doses, has no effect, probably because of reflex activation of the sympathetic system secondary to the hypotensive effect of the drug (615). Autonomic reflex activation also plays a prominent role in conscious animals, where, in contrast to anesthetized animals, hypotensive doses of verapamil, nifedipine, diltiazem, and felodipine produce marked increases in heart rate, which are attenuated by propranolol and atropine (e.g., 86, 805). Moreover, some calcium entry blockers may exert inhibitory effects on arterial baroreceptors (494, 1055).

b. CONTRACTILITY. In isolated preparations, calcium channel blockers exert negative inotropic effects that appear to be directly related to blockade of slow inward current (see above), or to reduction of the rise in cytosolic Ca^{2+} (aequorin signal) (774). For instance, quantitative analysis with nifedipine has shown that IC_{50} values are of the same magnitude for inhibition of contractility and Ca^{2+} current (about 0.5 μM). The order of potency is nifedipine > verapamil > diltiazem (55, 760). The inhibitory effects can be antagonized by increasing extracellular Ca^{2+} concentration (328). In agreement with the use-dependence observed for verapamil in electrophysiological studies (see section III B 2), the negative inotropic potency of this drug increases with the rate of stimulation (52, 810). In contrast, the negative inotropic action of nifedipine is frequency independent (56). The use-dependent inhibition by verapamil has been recently confirmed on human heart tissue by Ferry et al. (277). The inhibitory behavior of verapamil or D 600 is thus clearly different from that of inorganic Ca^{2+} blockers and is not mimicked by Ca^{2+} withdrawal (fig. 7).

Barry et al. (47) have recently studied the action of six calcium antagonists on contractility and calcium fluxes in cultured mycoardial cells prepared from chick embryos. They have observed that the order of negative inotropic potency was: nifedipine; D 600; lidoflazine > verapamil; diltiazem > perhexiline. Equipotent negative inotropic concentrations of verapamil, D 600, perhexi-

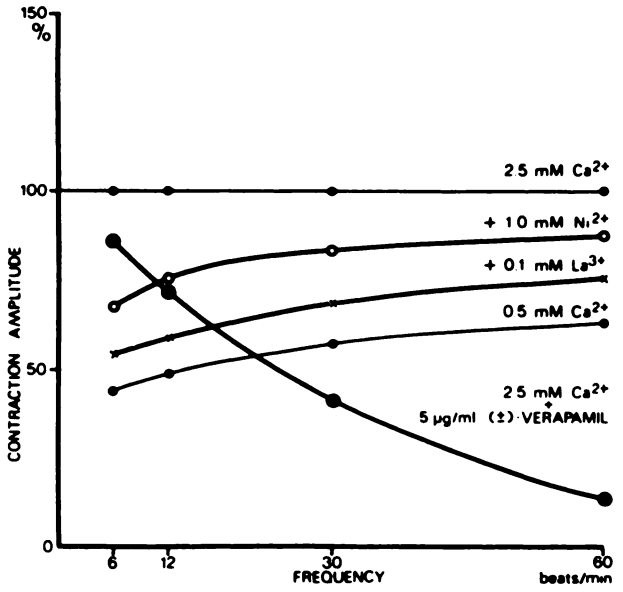

FIG. 7. Effect of verapamil and of di- or trivalent cations on steady-state amplitude-frequency relationship of isotonically contracting cat papillary muscle. Amplitudes of contractions under control conditions (2.5 mM Ca^{2+}) were set to 100% at each frequency of stimulation. Addition of Ni^{2+} or La^{3+} and reduction of extracellular Ca^{2+} had qualitatively similar depressing effects, whereas the inhibitory action of verapamil increased with frequency of stimulation. Adapted from Bayer et al. (52).

line, diltiazem, and lidoflazine produced a similar inhibitory effect on ^{45}Ca uptake into cells, whereas nifedipine produced no significant inhibition of ^{45}Ca uptake. Because this uptake may occur through both slow calcium channels and Na^+-Ca^{2+} exchange, the effect observed could be explained by an action of verapamil and related drugs on a "slow" Na^+ channel; this could decrease Na^+ loading and Ca^{2+} influx via the Na^+-Ca^{2+} exchange mechanism. An action of nifedipine, restricted to slow Ca^{2+} channels, might not be sufficient to be detected as an effect on net ^{45}Ca uptake. These results show that conclusions drawn from contractile studies on postulated calcium movements in cardiac cells are of little value, since drugs considered to be calcium antagonists may affect net calcium movements through different mechanisms.

In the conscious dog (760), verapamil maintains a significant dose-dependent, negative inotropy which is little affected by the autonomic nervous system. Nifedipine, at high dose levels, produces only a transient negative inotropic action followed by a sustained positive inotropic effect, due to increased adrenergic activity. Diltiazem produces little inotropic effect in conscious dogs and is unaffected by autonomic blockade.

3. Pathophysiological models. Calcium antagonists are of wide therapeutic interest in various cardiovascular, pulmonary, and neurological diseases. A major question is whether or not their varied therapeutic effects are due to a same mode of action. Therefore, pathophysiological models are adjuncts to analysis of their mechanisms of action. In the cardiac field, the major models deal with the prevention of alterations evoked by anoxic or ischemic conditions. In in vitro and in vivo studies, it has been demonstrated that calcium antagonists prevent or reduce anoxic and ischemia-evoked alterations by a direct action on the cardiac cells and by an indirect effect, due to their action on the coronary arteries (403).

a. PATHOPHYSIOLOGY OF CARDIAC CELLULAR INJURY. Cardiac cellular injury leads first to reversible alterations in electrical and contractile properties followed by irreversible alterations and cell death (cardionecrosis). There are three main causes of cardionecrosis: ischemia (reduction of blood supply); anoxia (reduction of oxygen supply); and direct toxic action on the heart.

A striking and important characteristic of ischemia, and to a lesser extent of hypoxia, is its macroscopic and microscopic heterogeneity (573). Varying conditions of work load and tissue perfusion may create a transient or patchy ischemia. In the latter instance, islands of severely ischemic tissue may be interspersed with areas of normal tissue. This is well illustrated by the multifocal distribution of the cadionecrotic areas resulting from the injection of large doses of catecholamines into rats (437).

Within a few seconds of the onset of ischemia, there is a decline in contractile activity. This decline occurs at a time when excitability remains essentially normal (606). During these first few seconds, the available oxygen dissolved in the cytoplasm in anoxic or severely ischemic tissue will be utilized, and anaerobic conditions will develop within the cell (1163). Associated with this will be a major reduction or even complete abolition of oxidative metabolism, electron transport, and mitochondrial ATP production. Reduced mitochondrial metabolism will result in a rapid reduction in beta oxidation of fatty acids and a subsequent accumulation of fatty acyl-CoA derivatives. This accumulation may be exacerbated by cyclic AMP-mediated lipolysis of endogenous triglyceride, which itself may be triggered by the early, ischemia-induced release of catecholamines (846). The stimulation of glycolytic ATP production in the face of reduced mitochondrial activity (Pasteur effect) leads to the accumulation of lactate, which leaks from the cell (848).

An early feature of myocardial ischemia is the progressive development of intracellular acidosis. In addition to its inhibitory effect upon glycolytic activity and its possible role in early contractile failure, acidosis may contribute to the development of the later stages of ischemic damage.

The progressive evolution of ischemia is associated with the loss of intracellular ions, metabolites, and macromolecules. The loss of K^+ reflects the deficiency of the Na^+-K^+ pump and the activation of K^+ channels and is associated with electrical disturbances. The loss of metabolites, namely of adenine nucleotide precursors, reflecting gross alterations of membrane integrity, is of great importance as it reduces the possibility of restoration of cell function as the ischemic condition is reversed.

Blood levels of macromolecules such as creatine phosphokinase (CPK) may serve as indicators for the lesion and its size, but only large lesions can be detected by this means (492). The alteration of the membranes is associated with activation of phospholipases leading to stimulation of the arachidonic acid cascade and the formation of potent vasoconstrictor products.

The severity of myocardial alterations increases with the duration of ischemia, and reperfusion paradoxically accelerates and amplifies the ischemic damage. Similar observations have been made in anoxic or hypoxic conditions. The sudden readmission of oxygen also enhances cell necrosis. This phenomenon has been termed the "oxygen paradox" (491). The mechanism responsible for this paradoxical extension of tissue injury is unknown, but it has been proposed that calcium and mitochondria may play an important role in reperfusion damage, as during reperfusion cardiac mitochondria will preferentially use oxygen to support calcium transport instead of ATP production (878). Many of the characteristics of the damage seen with the oxygen paradox parallel those of the calcium paradox, the latter involving the reintroduction of calcium after a period of calcium-free myocardial perfusion (491).

b. EXPERIMENTAL MYOCARDIAL INFARCTION. Fitzpatrick and Karmazyn (286) have compared the influence of diltiazem, nifedipine, and low calcium media on the damage evoked by reoxygenation and reperfusion, quantified by measuring the amount of CPK released into the perfusion fluid. Diltiazem (10 μg/ml) produced a reduction by about two-thirds in enzyme release upon reoxygenation, yet at this concentration it had no influence on reperfusion-induced enzyme release. The reduction by diltiazem of CPK release during reoxygenation was dependent on the drug's presence during the hypoxic phase of perfusion, whereas low calcium (in reoxygenation and reperfusion) or nifedipine treatment (reperfusion) was protective irrespective of whether the treatment was present during hypoxia/ischemia or reoxygenation/reperfusion. The reduction in enzyme release was associated with an enhanced mechanical recovery and a reduction in arrhythmias. Enhanced coronary resistance and myocardial contracture associated with these ischemic and anoxic manipulations are also reduced when the enzyme leakage is reduced. There are differences in the ability of calcium antagonists to decrease injury, and, for a given drug, the beneficial effects depend on the type of insult and the time at which the drug is administered (202, 491, 813, 878).

Those protective effects observed in isolated hearts support the hypothesis that it might be possible to obtain similar protection in vivo in the case of myocardial infarction, and there are optimistic reports in the literature of the possibility of reducing infarct size with calcium antagonists after acute coronary artery occlusion. The limitations of experimental models have recently been discussed by Yellon et al. (1180), who have pointed out the importance of distinguishing between reducing and delaying ischemic injury. Falsely positive results may be claimed if the duration of the experimental observation is too limited. We will summarize below publications mainly dedicated to particular drugs.

i. Verapamil. In in vivo dog studies, it has been reported that verapamil protects the myocardium against ischemic conditions due to occlusion of coronary arteries, as shown by reduction of epicardial ST segment elevation (998) and reduction in the extension of myocardial necrosis (225, 899, 913). The protective effect of verapamil was thought to be due to its Ca^{2+} antagonist property and did not seem to be dependent upon alterations in heart rate, since it was observed in experiments where the heart was paced. Nor was it associated with significant changes in the hemodynamic function of the heart (982). Verapamil (100 μg/kg) injected 5 min before ligation of small coronary branches had no effect on the level of glycolytic intermediates (545).

Reduction of infarct size following acute occlusion of coronary arteries has been attributed to an improvement of the coronary collateral flow in the ischemic zone, enhancing oxygen supply, in association with a reduction in oxygen demand, due to the negative inotropic effect of verapamil (998, 1149). However, Karlsberg and coworkers (608) have reported that a slow verapamil infusion commenced 5 h after acute coronary occlusion did not induce a marked hypotension, and that, while it increased blood flow to normal myocardium, flow in the ischemic area was not improved, and infarct size was not reduced.

In bypass open chest surgery, inclusion of verapamil (0.5 $mg \cdot l^{-1}$) in the cardioplegic solution resulted in improved myocardial protection, preserving mitochondrial function by maintaining the state 3 of respiration and decreasing mitochondrial Ca^{2+} uptake (873).

In studies on isolated hearts, verapamil apparently improved the mechanical performance of the ischemic heart by decreasing the energy demand during ischemia, rather than by blocking Ca^{2+} influx during reperfusion. Indeed, verapamil (0.1 to 1 μM) reduced mitochondrial Ca^{2+} uptake during reperfusion or reoxygenation when it was perfused both prior to, and during, ischemia or anoxia, but not when it was added only during reperfusion or reoxygenation (98, 815, 817, 1132). In the hypoxic heart, verapamil reduced CPK release, maintained mitochondrial function by preventing calcium overload, and thereby attenuated the increase in resting tension (97, 98, 814).

ii. Diltiazem. In in vivo studies, diltiazem has been shown to lessen the effect of ischemia induced by acute coronary occlusion and to reduce infarct size when administered prior to occlusion (122, 165, 641). It increased myocardial blood flow in the ischemic region without concomitant myocardial depression (a typical observa-

tion with other Ca^{2+} antagonists) (759, 802). It also lowered tissue levels of lactate and free fatty acids (1141), preserved tissue levels of CPK (290) and high-energy phosphate intermediates (1185), and preserved mitochondrial function by preventing depression of the state 3 of respiration and mitochondrial Ca^{2+} overload (641, 743, 801, 1142). It reduced oxygen consumption by a direct effect, and indirectly by its negative inotropic or chronotropic effects. In open chest surgery (clamped aortic root), it afforded protection by reducing afterload, thus increasing cardiac output. It increased collateral flow and prevented Ca^{2+} overload (1121).

In isolated perfused hearts subject to ischemia, diltiazem improved the recovery of ATP and creatine phosphate during reperfusion (585), reduced purine (200) and lactate dehydrogenase (482) release, and enhanced the mechanical performance of the heart (544, 585). In hypoxic hearts diltiazem (1 to 10 μM) attenuated the development of anoxic contracture and improved contractility during reoxygenation (97). According to Hamm and Opie (482), diltiazem affords the best protection of cell integrity and mechanical function when compared to other available slow channel blockers.

iii. Nifedipine. In in vitro studies utilizing isolated perfused heart, nifedipine (30 to 300 nM) protected against ischemia by improving myocardial performance, increasing coronary flow, decreasing myocardial O_2 demand and ATP levels, and decreasing purine release and mitochondrial Ca^{2+} uptake (201, 486, 505, 585, 586).

In vivo, nifedipine protected the heart against regional ischemia in either closed chest (225, 506) or open chest animals (489, 979, 1140). It limited the extension of infarct size as measured enzymatically and histologically, but high doses induced an increased extension of the necrotic zone, manifested by electrocardiogram (ECG) changes and an augmentation and earlier appearance of CPK in coronary veins (979). It reduced early postinfarction ventricular fibrillation when given 15 min before ligation of left coronary artery (258), but not when given 20 min after ligation (912). Even in vivo, nifedipine protected the heart at doses which had no significant hemodynamic actions. However, in the baboon, nifedipine infused i.v. (30 $\mu g \cdot kg^{-1} \cdot h^{-1}$) for 1 h before occlusion of coronary artery did not improve the histological appearance after reperfusion (372).

Some authors (489, 1140) have claimed that nifedipine could protect ischemic heart by a direct effect on cardiac cells, since they found that it did not affect blood flow in the ischemic zone, but increased flow in the nonischemic zone. On the other hand, Henry et al. (507) concluded that the protective effect was due to an action on flow to the ischemic zone, through a Ca^{2+} influx inhibitory effect. Nifedipine did not greatly affect changes in myocardial carbohydrate metabolism caused by coronary artery ligation (545).

In open chest heart surgery, nifedipine has been used as a cardioprotective agent and as an adjunct to hypothermia and cardioplegia. The infusion of nifedipine alone does not arrest the heart but, when combined with hypothermia, it stops the heart, due to its inhibitory effect on Ca^{2+} influx, an effect that could persist during the initial few minutes of reperfusion (when a rapid influx of Ca^{2+} occurs) (164).

iv. Lidoflazine. Lidoflazine is known to be beneficial in the treatment of ischemic heart disease (199) and of angina pectoris (272, 572). In vascular smooth muscle, it antagonizes both normoxic and anoxic vascular contractions and augments adenosine-induced relaxation (1107). It reduces systemic vascular resistance with a subsequent drop in blood pressure (293). In the myocardium, it has a minor negative inotropic effect (1109), but a significant negative chronotropic effect, reducing both resting and exercise-dependent increases in heart rate (199). It inhibits myocardial contracture evoked by strophanthidine (128), reverses atrial fibrillation to sinus rhythm, and reduces the frequency of ventricular ectopic beats following coronary artery ligation (169). It markedly enhanced myocardial tolerance of ischemia in experimental animal studies (292), as shown by preservation of high energy phosphate content, of cell ultrastructure, and of hemodynamic function and by prevention of the accumulation of Ca^{2+} into cellular organelles. Lidoflazine, like the other Ca^{2+} entry blockers, appears to be a useful adjunct to cardioplegic solutions (611).

In isolated perfused rabbit heart, lidoflazine (4 $mg \cdot l^{-1}$) administered prior to ischemia (45 min) and/or during reperfusion improved the mechanical performance and decreased the release of lactate dehydrogenase, lactate, and purines (966). When lidoflazine treatment was started 5 min after the onset of reperfusion, however, the drug was ineffective. Nayler (812) reported that lidoflazine protected mitochondria against the effect of ischemia and reperfusion. Mitochondrial Ca^{2+} overload was reduced and the ATP production capacity maintained with an improvement in mechanical performance.

v. Other dihydropyridines. The action of nicardipine has been examined in several models of myocardial infarction by Alps et al. (18). These authors have reported that, in a dog model of partial myocardial ischemia, elevations in the ST segments of epicardial ECGs were inhibited by nicardipine over a cumulative i.v. dose range of 1 to 20 μg/kg. They observed that dogs dosed p.o. with 1 to 2 mg of nicardipine per kg daily for 16 wk that survived a 1-wk occlusion of the left anterior descending coronary artery (LAD) developed a superior coronary collateral circulation compared with untreated animals. Interestingly, this observation is a confirmation of the report by Schaper et al. (960), who as early as 1966 obtained similar results with lidoflazine.

Wartier and Gross (1127) have compared changes in coronary collateral flow produced by nitrendipine, nifedipine, and diltiazem after occlusion of the left anterior

coronary artery. They observed that the three calcium antagonists, given by i.v. perfusion, produced an increase in flow distal to the chronic coronary occlusion. This increase was dose dependent, and differences were apparent in the regional effects of the drugs: diltiazem produced a selective increase in subendocardial perfusion; the dihydropyridines produced an increase in subepicardial perfusion within the collateral-dependent zone without any change in subendocardial flow until aortic pressure was returned to predrug levels.

Nisoldipine (20 to 300 nM) also prevented changes evoked by ischemia in isolated perfused rat hearts (202).

c. PROTECTION AGAINST THE CARDIONECROTIC ACTION OF ISOPROTERENOL. The cytoprotective effect of calcium antagonists against cardionecrosis induced in rats by injection of large doses of isoproterenol has also been demonstrated (403, 436). When a dose of isoproterenol (30 mg/kg) was injected s.c. and the animals were killed 24 h later, histological examination showed necrosis of muscle fibers disseminated in the myocardium, with a preferential localization at the apex of the heart (437). This toxicity is characterized by a reduction in high energy phosphate content and a large gain in the calcium content of the myocardium. One of the most characteristic properties of calcium antagonists is their ability to protect the myocardium against this injury. This protection has been documented with verapamil (303), cinnarizine, flunarizine, lidoflazine (423), diltiazem, and dihydropyridines (303). Inhibition by verapamil of isoproterenol cardionecrosis could not be explained by a beta-antagonistic effect (303, 816).

d. EXPERIMENTAL ARRHYTHMIAS. Kaumann and Aramendia (612) induced ventricular fibrillation by ligation of the left anterior descending coronary artery and observed that i.v. injection of verapamil (0.79 mg/kg) prevented ventricular fibrillation in dogs, whether or not they were pretreated with reserpine. This antifibrillatory effect has been confirmed in different laboratories (337, 912, 1056). Verapamil also antagonizes the reduction of ventricular fibrillation threshold during ischemia. Similar protective effects have been observed with other calcium antagonists, such as D 600, diltiazem (1056), dihydropyridines (258), and bepridil (66, 721). It has been proposed that this action is due to a lessening of ischemic insult rather than to a primary electrophysiological phenomenon, but this hypothesis has been questioned by Clusin et al. (165). Fleckenstein and coworkers (303, 656) reported that verapamil and related drugs improve the myocardial utilization of high-energy phosphates. However, it has been shown by Thandroyen (1056) that the antifibrillatory effect of calcium antagonists is not contingent upon improvement of myocardial energetics.

Curtis et al. (186) have studied the action of verapamil on arrhythmias induced by coronary occlusion and by electrical stimulus in conscious and pentobarbitone-anesthetized rats. They have shown that the ECG alterations were corrected in a quite different way by verapamil and quinidine, suggesting that the antiarrhythmic action of verapamil is not related to fast channel blockade, but could well be due to slow channel blockade. Indeed, plasma concentrations of verapamil needed to reduce arrhythmias are close to IC_{50} values for inhibition of contractility in rat atria and ventricles. Similar concentrations depress action potentials induced by isoproterenol in K^+-depolarized ventricles. As was recently shown by Brown et al. (116), those action potentials are Ca^{2+} mediated and show a very high sensitivity to the inhibitory action of calcium entry blockers and a very low sensitivity to quinidine.

Besides slow action potentials, oscillatory after-potentials might be potentially important in the genesis of acute ischemic ventricular arrhythmias. There are differences in the actions of calcium antagonists on those two abnormalities. Amerini et al. (19) compared, in sheep cardiac Purkinje fibers, the effects of verapamil and diltiazem on normal action potentials, abnormal automaticity at depolarized membrane potentials, and oscillatory after-potentials. They observed that verapamil and diltiazem (in the micromolar range) affect action potentials in normal Purkinje fibers differently. They block the abnormal automaticity at depolarized membrane potential with a similar efficacy, but diltiazem is more effective than verapamil in delaying the development of oscillatory after-potentials induced by strophantidin. Verapamil abolished barium-induced oscillatory after-potentials but caused the appearance of spontaneous activity, while diltiazem had no such effect. These differences could be attributed to differences in K^+ conductance, it being reduced by verapamil (610) but increased by diltiazem (770).

Such differences between electrophysiological properties of calcium antagonists have been found in clinical as well as in experimental studies. In excised rabbit atrioventricular node, diltiazem, verapamil, and nifedipine suppress conduction and prolong the refractory period. Clinically, diltiazem and verapamil exert a similar suppressive effect on the AV node and are useful for treating and preventing AV nodal reentrant tachycardia. Nifedipine in clinically practical doses has no antiarrhythmic properties, probably because of reflex activation of the sympathetic system secondary to its hypotensive effect which is greater than that of the other two calcium antagonists (615). Paroxysmal supraventricular tachycardias are usually associated with reentrant circuits involving the atrioventricular node and/or bypass tracts. Verapamil, a potent agent used to control supraventricular tachycardia, acts by inhibition of one of the reentrant circuits (849).

e. THE MECHANISM OF CARDIOPROTECTION BY CALCIUM ANTAGONISTS. The complexity of the factors involved in cardiac injury evoked by anoxia and ischemia

makes difficult the identification of one single initial biochemical lesion. Furthermore, in living animals and in patients, it is difficult to identify the primary site of action for the protective effect: vessels or myocardium? or both?

A vascular smooth muscle effect involves an attenuation of the vasospastic response to agents released during the pathological aggression. There is, indeed, a vicious cycle involving anoxia and ischemia. Anoxic alterations in the cellular membrane stimulate a release of fatty acids (2) and activate the arachidonic acid cascade. Active metabolites (e.g., prostaglandins) are not only responsible for early dysrhythmias, but also for coronary vasospasm which, in turn, induces an ischemic aggression that augments the consequences of anoxia. Blockade of this vicious cycle may be obtained by a reduction of the vasospastic response and, in experiments on isolated hearts, effects on the vasculature are usually seen at lower concentrations of calcium antagonists than are effects on the myocardium. For instance, De Jong et al. (202) have shown that nisoldipine inhibits the efflux of purines from the ischemic heart at concentrations 50 times higher than those active on coronary flow. There is a controversy regarding the mechanism responsible for this effect. Some authors (202, 813) believe that the protection could be due to a reduction of the contractile behavior due to reduced calcium influx. Other authors favor the idea of an intracellular action of calcium entry blockers on phosphodiesterase (830) or on Na^+-Ca^{2+} exchange at the mitochondrial level (759, 975).

Some products of the arachidonic acid cascade, i.e., thromboxane A_2, may contribute to the postischemic "no-reflow phenomenon," not only by inducing vascular smooth muscle contraction, but also by producing endothelial cell damage (195). Flunarizine, niludipine, and nimodipine have been shown to protect vascular endothelium against the deleterious effects of thromboxane A_2 (195).

D. Nervous Tissues

1. Ionic currents. a. CALCIUM CHANNELS IN NEURONES. Calcium channels in neurones have been extensively studied by electrophysiological techniques (478, 1078). Almost all nerve cell bodies possess both sodium and calcium channels, but the ratio of the magnitude of the two voltage-dependent currents (I_{Na} and I_{Ca}) varies widely. Calcium currents in neurones may control somatic and axonal spike configurations and discharge frequencies via calcium-activated potassium currents. Calcium currents may be responsible for impulse propagation in dendrites. At nerve terminals, voltage-sensitive calcium channels are essential for the initiation of transmitter release.

In many respects, neuronal calcium channels are similar to their counterparts in other cell types. The Ca^{2+} current of neurones saturates as the external Ca^{2+} concentration is increased, indicating the existence of a Ca^{2+} binding site within the channel. The affinity sequence at this site for permeant cations is $Ca^{2+} > Sr^{2+} > Ba^{2+}$. Several di- or trivalent cations compete with Ca^{2+} for the channel binding site, but they are unable to pass through the channel. These inorganic blockers include La^{3+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, and Mg^{2+}.

During maintained depolarization, the Ca^{2+} conductance usually declines. This inactivation phenomenon is extremely variable according to the cellular system investigated. The hypothesis has been put forward that inactivation may result from accumulation of intracellular Ca^{2+}, which inhibits calcium conductance. In snail neurones, calcium channel inactivation seems to be voltage dependent as well as $Ca^{2+}{}_i$ dependent (112). Little inactivation has been found so far for calcium channels involved in excitation-secretion coupling [squid synaptic terminals (692) and adrenal chromaffin cells (271)].

The diversity of the kinetic characteristics of calcium currents in various neuronal systems may result from the coexistence in variable proportions of different types of calcium channels. Indeed, some neuronal cells have been shown to possess at least two distinct types of calcium conductance (765). Three subtypes (L, T, and N) have been described by Nowycky et al. (835) in chick dorsal root ganglion cells. Their features will be examined in section III B.

b. EFFECTS OF CALCIUM ENTRY BLOCKERS ON IONIC CURRENTS AND ACTION POTENTIALS. This topic has been reviewed by Miller and Freedman (766) and by Louvel et al. (697). Usually, phenylalkylamines (verapamil, D 600) block I_{Ca} or calcium spikes only at rather high concentrations (>10 or 100 μM). Inhibition of I_{Na} or sodium spikes (but not potassium currents) generally occurs over a still higher concentration range. However, Baker et al. (38) noted that in squid axon I_{Na} was reduced by concentrations of D 600 that inhibited Ca^{2+} entry through the "late" calcium channel. Especially puzzling are the results obtained by Douglas and Taraskevich (224) on cells from rat pars intermedia: verapamil or D 600 (100 μM) reduced sodium spikes, while leaving calcium spikes unaffected. Neuronal calcium channels are about equally sensitive to diltiazem and to verapamil (10, 558, 559). Diltiazem appears to inhibit calcium current competitively in *Helix* neurones (10).

The effects of dihydropyridines have been recently discussed by Miller (765). Nifedipine, nicardipine, or nitrendipine at concentrations of 1 μM or more did not modify calcium spikes in a variety of cells (224, 659, 839, 1054), but nifedipine (6 μM) reduced sodium spikes in pars intermedia cells (224). Nifedipine (10 μM) had no effect on I_{Ca} in chick cultured ciliary neurones or in rat superior cervical ganglion cells (471). However, nifedipine depressed I_{Ca} in *Helix* neurones (IC_{50} 3 μM) (827) and in *Aplysia* neurones (IC_{50} 1 μM) (471). In rabbit

nodose ganglion C-cells, nifedipine (as well as verapamil and diltiazem) reduced Ca^{2+} spikes elicited in Na^+-free solution (IC_{50} between 1 and 10 μM) (559). Action potentials were modified in normal physiological solution, indicating depression of Na^+ influx and of K^+ efflux. Flunarizine (2 to 3 μM) and nifedipine (1 μM) depressed action potentials in hippocampal dentate granule cells (393, 394). Brown et al. (114) reported that, in hippocampal neurones, a noninactivating I_{Ca} could be depressed by nimodipine (IC_{50} 1 μM) and increased by Bay K 8644. In a recent study by the patch-clamp technique (cell-attached mode), Nowycky et al. (834) also found that the noninactivating current (L-type channel) of chick dorsal root ganglion cells in culture was increased by Bay K 8644 (5 μM). In contrast, T- or N-type channels did not respond to Bay K 8644 (835). In the same type of cells, Boll and Lux (85) found that a fast inactivating calcium current was insensitive to nifedipine and verapamil, whereas a slowly inactivating current was depressed by verapamil, but not by nifedipine. Paradoxically, nifedipine frequently produced agonistic effects on the slowly inactivating current.

In conclusion, few neuronal calcium conductances appear to be affected by dihydropyridines, and these dihydropyridine-sensitive conductances have been demonstrated almost exclusively in nonmammalian systems. Moreover, dihydropyridines (and other calcium channel blockers) may affect other ionic conductances at similar concentrations.

2. Neurotransmission and synaptosomal fractions. a. NORADRENERGIC TRANSMISSION IN HEART AND SMOOTH MUSCLE TISSUES. **i. Heart.** Haeusler (475) found no effect of verapamil at a concentration of 2 μM on norepinephrine release from cat isolated heart produced by electrical stimulation, acetylcholine stimulation, or K^+-depolarization. On the other hand, using rabbit heart, Göthert et al. (455) reported that verapamil competitively inhibited (K_i 9 μM) norepinephrine release evoked by Ca^{2+} in K^+-depolarized preparations. Verapamil, D 600 and prenylamine showed similar potencies on K^+-depolarized tissue (IC_{50} 10 to 20 μM) and were more potent on acetylcholine-induced release (IC_{50} 1 to 2 μM). Verapamil was distinctly less potent on norepinephrine release evoked by nerve stimulation (IC_{50} 70 μM). In the experiments of Göthert et al. (455), the drugs did not affect the basal output of norepinephrine. In contrast, in isolated rat atria preloaded with [^{3}H]norepinephrine, verapamil (>1 μM) enhanced the spontaneous release of radioactivity in a calcium-independent manner (149). The additional outflow induced by verapamil consisted mainly of norepinephrine metabolites. Thus, phenylalkylamines influence noradrenergic transmission in heart at concentrations that are higher than those required to inhibit contractility, and they may exert a reserpine-like action on noradrenergic terminals.

Nifedipine (1 μM) depressed norepinephrine outflow induced by nerve stimulation in canine heart, but again the mechanical activity was more sensitive to this drug than was neurotransmitter release (1020).

The mammalian heart releases norepinephrine during periods of ischemia and reperfusion. Nayler and Sturrock (818) reported that this release was blocked by relatively low concentrations of verapamil (0.3 to 1.2 μM) or diltiazem (1.3 μM) and that (−)verapamil was the active isomer. The mechanism of this protective effect remains unclear.

ii. Smooth muscle tissues. Norepinephrine release is not affected by concentrations of inhibitors that completely block smooth muscle calcium channels. At a concentration of 3 μM, nifedipine did not modify K^+-evoked release from rabbit basilar and facial arteries (532), or release evoked by electrical stimulation of rabbit urethra (666). When [^{3}H]norepinephrine-loaded tissues are treated with high concentrations of drugs, the most general effect is a paradoxical increase of spontaneous tritium outflow. This phenomenon has been observed in rabbit ear artery treated with 10 μM flunarizine, 10 μM nicardipine, or 30 μM diltiazem (592); in dog saphenous vein treated with 3 to 30 μM verapamil, diltiazem, or nicardipine (1048); in rabbit aorta treated with verapamil, D 600, diltiazem, or prenylamine at concentrations higher than 10 μM (604); and in rat tail artery and guinea pig vas deferens treated with 10 to 100 μM verapamil (1189). Kajiwara and Casteels (592) found that the enhanced tritium efflux consisted essentially of deaminated metabolites, whereas Karaki et al. (604) reported that the release of [^{3}H]norepinephrine was also increased. This efflux was calcium independent (604). Yohimbine neither mimicked nor prevented the action of verapamil, indicating that presynaptic alpha-adrenoceptors were not involved (1189). In view of this prominent reserpine-like action of calcium entry blockers on spontaneous tritium release, possible effects on additional release evoked by K^+-depolarization or electrical stimulation are very difficult to assess, and both stimulatory and inhibitory effects have been reported. In rabbit pulmonary arterial strips preincubated with [^{3}H]norepinephrine, 15 μM diltiazem did not influence basal tritium release, but reduced the tritium outflow evoked by a 8-Hz electrical stimulus or by K^+-depolarization (1188). However, no inhibition of tritium outflow was observed at lower stimulation frequencies.

Recently, Cena et al. (139) examined the effect of Bay K 8644 in rat vas deferens preloaded with [^{3}H]norepinephrine. Bay K 8644 (1 μM) did not affect the spontaneous release of tritium, nor the calcium-independent release evoked by tyramine, but increased the calcium-dependent release elicited by K^+-depolarization or electrical field stimulation. Phentolamine (10 μM) also increased the release of tritium evoked by potassium or electrical stimulation. In the presence of phentolamine, Bay K 8644 did not produce any further effect on tritium release, which suggests that it acted via presynaptic calcium channels modulated by alpha$_2$-receptors.

Excitatory junction potentials (EJPs) evoked by nerve stimulation are also quite resistant to calcium entry blockers. Several dihydropyridines were inactive at a concentration of 0.2 μM in rabbit mesenteric artery (705), and nifedipine (30 μM) did not influence EJPs in vas deferens (76). However, nicardipine reduced the amplitude of EJPs in rabbit ear artery (>10 μM) (592) and guinea pig basilar artery (3 μM) (356). Diltiazem inhibited EJPs in guinea pig vas deferens (10 μM) (1043) and mesenteric artery (50 μM) (1038) and in rabbit ear artery (30 μM) (592). Inhibitory effects were also noted for verapamil (10 μM) and bepridil (5 μM) in guinea pig mesenteric artery (1187) and for flunarizine (>10 μM) in ear artery (592). In the ear artery (592), calcium entry blockers probably depressed EJPs by a direct action on the smooth muscle membrane, since they did not concomitantly reduce norepinephrine release at nerve terminals. In this regard, there is a possibility that some of the drugs act as receptor antagonists in the concentration ranges tested (see section III D).

b. CHOLINERGIC TRANSMISSION IN SKELETAL MUSCLE AND INTESTINAL SMOOTH MUSCLE. Neuromuscular transmission in skeletal muscle is little affected by calcium entry blockers. In an in situ canine tibial muscle preparation, nifedipine, diltiazem, or verapamil injected into the femoral artery potentiated the twitch tension evoked by nerve stimulation, but not contractions produced by direct stimulation (957). Similar results were obtained by Asai et al. (31) with a phrenic nerve-diaphragm muscle preparation. Verapamil stereoisomers were equiactive. Nitrendipine (20 μM) did not impair diaphragm contraction evoked by stimulation of the phrenic nerve (259). End plate potentials elicited by nerve stimulation were not modified by high concentrations of verapamil or D 600 (31, 106, 454, 799, 1097). Again, paradoxical calcium-independent increases in the frequency of spontaneous miniature end plate potentials (MEPPs) were observed with verapamil or D 600 (31, 454, 885) and with diltiazem (106).

In the guinea pig ileal myenteric plexus preparation, D 600, nicardipine, and diltiazem (10 μM) did not affect acetylcholine release produced by nerve stimulation, while abolishing twitch responses (600).

c. NEUROTRANSMITTER RELEASE FROM BRAIN TISSUE. The first experiments demonstrating a key role for Ca^{2+} in the functioning of nerve endings were conducted on isolated neurohypophysis (222, 223). In this system, D 600 inhibited ^{45}Ca uptake and oxytocin release elicited by K^+-depolarization with an IC_{50} of about 10 μM (228). D 600 and prenylamine inhibited vasopressin release in response to electrical stimulation, and the action of D 600 was antagonized by elevating external Ca^{2+} (939). However, D 600 (20 μM) also abolished secretion evoked by Ca^{2+} in neurohypophyses treated with the Ca^{2+} ionophore A 23187 (921).

Verapamil and D 600 inhibited (IC_{50} 10 to 20 μM) K^+-depolarization-evoked tritium efflux from rat brain slices preloaded with [^{3}H]norepinephrine, gamma-[^{3}H]aminobutyric acid ([^{3}H]GABA) (839, 1114), or [^{3}H]serotonin (993). Nicardipine (1 μM) did not affect the K^+-evoked release of [^{3}H]GABA (839). At a concentration of 100 μM, verapamil increased spontaneous [^{3}H]serotonin release (793). In slices from rabbit caudate nucleus preincubated with [^{3}H]choline or [^{3}H]dopamine, verapamil (10 μM), diltiazem (100 μM), and the dihydropyridine ryosidine (10 μM) slightly inhibited tritium release evoked by electrical stimulation, K^+-depolarization, or glutamate; in slices preloaded with [^{3}H]dopamine, verapamil and diltiazem also enhanced the basal efflux of tritium (1021). In rat striatal slices, verapamil and flunarizine (10 μM) did not modify basal dopamine release, but inhibited by about 50% the K^+-stimulated release (459). Flunarizine also depressed K^+-stimulated ^{45}Ca uptake.

In rabbit hypothalamic slices preloaded with [^{3}H]norepinephrine, verapamil (1 to 10 μM) increased tritium release evoked by electrical stimulation, whereas diltiazem (10 to 100 μM) increased basal efflux of tritium, but did not affect the release evoked by electrical stimulation (365). Verapamil also antagonized the inhibitory effect of alpha$_2$-agonists on tritium overflow elicited by electrical stimulation. Thus, Galzin and Langer (365) concluded that verapamil enhanced [^{3}H]norepinephrine release evoked by electrical stimulation through an inhibitory action at presynaptic alpha$_2$-receptors. Phenylalkylamines indeed displaced alpha-adrenoceptor ligands from rat brain membranes at relatively low concentrations (section III D; refs. 34, 260, 391, and 819).

Louvel et al. (697) examined the effect of calcium channel blockers on extracellular Ca^{2+} in electrically stimulated hippocampal slices by means of Ca^{2+} electrodes. They concluded that nifedipine (1 μM) had no effect on presynaptic Ca^{2+} entry, while reducing postsynaptic calcium conductances.

Recently, Middlemiss and Spedding (751) and Middlemiss (750) made the interesting observation that Bay K 8644 moderately enhanced tritium release from rat brain slices preincubated with [^{3}H]serotonin, [^{3}H]norepinephrine, and [^{3}H]choline, and depolarized by 25 mM KCl. The additional stimulatory effect of Bay K 8644 was antagonized by nifedipine, diltiazem, verapamil, or flunarizine, whereas, at a concentration of 1 μM, these drugs did not inhibit the tritium efflux elicited by K^+-depolarization alone. The authors concluded that Bay K 8644 induces an activated state of presynaptic calcium channels and that this particular state is sensitive to calcium entry blockers.

Stimulation of polyphosphoinositide breakdown and subsequent activation of protein kinase C may play a role in neurotransmitter release (1123, 1190). Kendall and Nahorski (627) found that K^+-depolarization of cerebral cortical slices enhanced inositol phospholipid hydrolysis, and that this effect was potentiated by Bay K 8644 (0.1 to 10 μM). PN 200-110 inhibited in a stereose-

lective manner the response to K^+-depolarization and to Bay K 8644.

d. SYNAPTOSOMAL FRACTIONS. As first shown by Nachshen and Blaustein (799), Ca^{2+} entry into synaptosomes and the subsequent neurotransmitter release are quite resistant to the action of calcium entry blockers. Half-maximal inhibition of K^+-depolarization-evoked ^{45}Ca uptake usually requires concentrations of verapamil or D 600 of 5 to 10 μM or more. However, Norris and Bradford (832) reported that depolarization-stimulated ^{45}Ca uptake (high-potassium or veratridine) was completely blocked by 0.5 μM verapamil (while being unaffected by 0.1 μM verapamil). According to Nachshen (798), the potency of verapamil was significantly enhanced when synaptosomes were depolarized briefly (0.1 to 1 s) in the presence of the drug before addition of ^{45}Ca. Inhibition of ^{45}Ca uptake by verapamil was noncompetitive (799). However, inhibition of [^{3}H]norepinephrine release from brain vesicular preparations by D 600 or diltiazem was antagonized by increasing the Ca^{2+} concentration (234).

Blockade of ^{45}Ca entry into depolarized synaptosomes by relatively high concentrations of phenylalkylamines may result from nonspecific membrane effects. Indeed, inhibition by D 600 is not stereoselective (Wibo, unpublished results). Verapamil and D 600 inhibit the uptake of serotonin, dopamine, and norepinephrine by synaptosomes over a comparable concentration range (IC_{50} 2 to 30 μM) (736). They interact with alpha-adrenoceptors (section III D) and with brain muscarinic receptors (260). Nevertheless, veratridine-activated sodium channels are less sensitive to verapamil than are calcium channels, (799, 832).

Most studies reported so far have shown that synaptosomal ^{45}Ca uptake is insensitive to dihydropyridines (28, 191, 234, 488, 799, 839), including Bay K 8644 (892). However, Mendelson et al. (742) found that nitrendipine or nifedipine (1 μM) blocked the additional ^{45}Ca uptake evoked by diazepam (1 μM) in K^+-depolarized synaptosomes. More recently, Turner and Goldin (1084) reported that nitrendipine and nifedipine inhibited (IC_{50} 60 nM) a portion (around 20%) of the K^+-evoked ^{45}Ca uptake in their synaptosomal preparations. Their potency was strikingly increased in the absence of sodium ions (IC_{50} 2 to 4 nM).

Depolarization-dependent ^{45}Ca uptake by isolated nerve endings has generally been considered as reflecting Ca^{2+} entry via voltage-sensitive channels. Nachshen and Blaustein (800) resolved the ^{45}Ca influx evoked by K^+-depolarization into two components: a La^{3+}-sensitive influx that inactivated within 1 s; and a La^{3+}-resistant influx that did not inactivate within 1 min. The two components did not differ in their sensitivity to D 600. Part of the depolarization-dependent ^{45}Ca influx might plausibly be attributed to a Na^+-Ca^{2+} antiporter (1084, 1155). Indeed, Na^+-Ca^{2+} exchange being reversible and electrogenic [1 Ca^{2+}/3 Na^+; reviewed by Reuter (905)], depolarization should stimulate entry of Ca^{2+} associated with efflux of Na^+.

3. Cultured neuronal cells. Like synaptosomal fractions, some clonal cell lines of neuronal origin (PC12, NCB-20) and cultured cells from chick embryonic neural retina take up ^{45}Ca at a faster rate when incubated in high potassium or with veratridine. But, in contrast to synaptosomal fractions, depolarized cells are characterized by a remarkable sensitivity to dihydropyridine calcium entry blockers, the IC_{50} values for inhibition of K^+-stimulated ^{45}Ca uptake (0.6 to 10 nM) being close to the values reported for inhibition of smooth muscle contractility (350, 1046, 1063). With NCB-20 cells, inhibition of calcium uptake has been shown to be stereoselective, (−)nimodipine being 10 times more potent than (+)nimodipine, and (−)verapamil 4 times more potent than (+)verapamil (350). Inhibition of ^{45}Ca uptake or neurotransmitter release by dihydropyridines and displacement of bound [^{3}H]nitrendipine occur over similar concentration ranges (1046, 1063).

Ogura and Takahashi (839) reported that calcium spikes in PC12 cells were not blocked by nicardipine (1 μM) even upon repetitive stimulation, whereas this drug potently inhibited K^+-stimulated ^{45}Ca uptake in the same cells. The authors suggested the coexistence in PC12 cells of two independent pathways for voltage-dependent Ca^{2+} entry: a dihydropyridine-insensitive spike-generating channel and a dihydropyridine-sensitive channel with sustained activation state. The apparent insensitivity to dihydropyridines of other K^+-depolarized neuronal preparations (brain slices, synaptosomal fractions) remains puzzling, however.

In PC12 cells, the calcium "agonist" Bay K 8644 enhanced the Ca^{2+} dependent [^{3}H]norepinephrine release evoked by K^+-depolarization, and this effect was antagonized by nitrendipine (13). As shown by Freedman and Miller (351) on NG108-15 (neuroblastoma-glioma) cells, Bay K 8644 (as well as CGP 28392) markedly potentiated depolarization-induced ^{45}Ca influx, without affecting uptake by polarized cells. The Bay K 8644 enhancement of uptake was inhibited competitively by nitrendipine, but noncompetitively by D 600. Half-maximal stimulatory effects of Bay K 8644 were observed at concentrations of 10 to 30 nM. The marine toxin, maitotoxin, also activates calcium channels in cultured cells, and its action is antagonized by low concentrations of dihydropyridine inhibitors (352, 1047).

4. Behavior and pathophysiological models. Dihydropyridine calcium entry blockers have been shown to exert neuro- and psychopharmacological effects at relatively high doses. Nimodipine influenced the extrapyramidal system, aggressive defensive behavior, and chemically induced seizures (528), and nifedipine blocked sleep induction by flurazepam in the rat (742). At comparatively low doses (1 to 2 mg/kg), Bay K 8644 elicited marked

behavioral effects in mice (e.g., ataxia, decreased motor activity) and impaired rotarod performance (84). These effects were antagonized by nifedipine at doses that were inactive when given alone. Interestingly, these results of Bolger et al. (84) parallel those of Middlemiss and Spedding (751) on the interactions of Bay K 8644 and nifedipine with serotonin release from cortex slices.

Several calcium entry blockers (flunarizine, dihydropyridines) are effective cerebral protecting agents in models of brain hypoxia, ischemia, or metabolic intoxication (230, 528, 1111, 1133, 1134), and in experimental models of epilepsy (212, 528, 529, 1134). Verapamil, which shows little activity in vivo, counteracts the fall in extracellular Ca^{2+} concentration that occurs when brain tissue is submitted in vitro to ischemic conditions (477). It is not clear, therefore, to what extent brain protection may be attributed to direct effects on neurones, or to indirect effects via an action on cerebral blood vessels (622). Prevention of neuronal calcium overload might play a role in protection against hypoxic/ischemic damage (90, 1112). Blockade of neuronal calcium channels might be involved in the inhibition of seizure spread (1096).

E. Endocrine Tissues

1. Adrenal medulla. Adrenal chromaffin cells share many functions with sympathetic neurons, since they are able to synthesize, store, and release catecholamines. The content of catecholamine storage granules is delivered to the blood by an exocytotic process, triggered by the elevation of intracellular Ca^{2+} (642, 933). Hydrolysis of plasma membrane polyphosphoinositides and subsequent activation of protein kinase C by diacylglycerol may also be an essential step in exocytosis (643, 1152).

The physiological control of secretion relies on cholinergic innervation. Acetylcholine acts mainly through nicotinic receptors, and its stimulatory effect requires the presence of extracellular calcium (221). The dependence on external Na^+ is more controversial (140). Plausibly, the binding of acetylcholine to its receptor promotes sodium influx via an associated channel. The resulting depolarization then activates voltage-sensitive calcium channels, producing Ca^{2+} entry and secretion. Some additional Ca^{2+} ions might enter through the channel linked to the nicotinic receptor (963). Voltage-sensitive sodium channels and associated action potentials may also play a role in secretion (631).

This picture of catecholamine secretion is partly based on the use of calcium entry blockers (29, 168, 632, 874, 875, 963). In perfused adrenal glands, half-maximal inhibition by verapamil or D 600 of catecholamine secretion evoked by high-K^+ or acetylcholine required a concentration of 10 μM (29) or higher (875). In isolated chromaffin cells, the IC_{50} of verapamil or D 600 on acetylcholine- or high-K^+-evoked secretion was in the range of 1 to 10 μM (632, 963), while these drugs did not influence Ca^{2+}-evoked exocytosis in adrenal medullary cells treated with the calcium ionophore A 23187 or made leaky by high-voltage discharges (37). Inhibition of carbamylcholine-evoked secretion by verapamil or diltiazem was antagonized by Ca^{2+} (954, 1122). However, phenylalkylamines should not be viewed as highly specific inhibitors of voltage-dependent Ca^{2+} channels in adrenal medullary cells. D 600 blocked nicotine-stimulated Na^+ uptake by these cells, though at concentrations that were about 10-fold higher than those blocking Ca^{2+} uptake (173). Moreover, D 600 competitively antagonized the stimulatory effects of veratridine on Na^+ and Ca^{2+} uptake and catecholamine secretion (K_i 10 to 20 μM). In contrast, verapamil (1 mM) did not influence catecholamine release induced by tyramine, salbutamol, or theophylline (168).

Cena et al. (140) showed that nitrendipine inhibited the secretory responses to nicotine or high-K^+ in cultured chromaffin cells. The IC_{50} for inhibition of K^+-depolarization-evoked secretion (21 nM) was somewhat lower than the IC_{50} for nicotine-induced secretion (400 nM). Comparable data on intact glands have not been reported. However, in perfused glands, Bay K 8644 was found to potentiate markedly catecholamine output in the presence of 18 mM KCl but not in the presence of normal or maximally effective K^+ concentrations (367). The drug also enhanced ^{45}Ca uptake by isolated cells. These effects of Bay K 8644 were antagonized by nitrendipine.

2. Adrenal cortex. a. ZONA FASCICULATA-RETICULARIS. Corticotropin (ACTH) stimulates steroidogenesis and cyclic AMP synthesis, and both effects require the presence of extracellular calcium (933). Verapamil (60 μM) inhibited both corticosterone production and increase in cyclic AMP in ACTH-stimulated cells isolated from rat zona fasciculata-reticularis (877). D 600 was also an effective blocker of ACTH action in Y-1 mouse adrenal tumor cells (1128). Thus, ACTH-stimulated secretion of glucocorticoids seems to depend on a rise of intracellular Ca^{2+}, but the exact locus of calcium action remains uncertain (757). However, in the study of Shima et al. (986), verapamil (50 μM) did not affect ACTH-evoked corticosterone release. In man, acute cortisol responses to ACTH and angiotensin II were not influenced by nifedipine (758).

b. ZONA GLOMERULOSA. Aldosterone secretion is controlled primarily by angiotensin II, potassium ions, and ACTH. While the action of ACTH on steroidogenesis is mediated, at least partly, by cyclic AMP, the two other modulators do not enhance cyclic AMP production (985). In contrast, calcium is an essential factor in the aldosterone response of zona glomerulosa cells to various stimuli (262, 342).

K^+ concentrations higher than 3 mM depolarize glomerulosa cells (730), thereby inducing Ca^{2+} entry via voltage-sensitive calcium channels and steroid secretion.

A recent study using quin-2 and a fluorescent indicator of membrane potential has elegantly confirmed this scheme (125). Half-maximal elevation of the cytosolic Ca^{2+} concentration (Ca^{2+}_i) and steroid output occurred at 6 to 7 mM K^+. These effects of K^+ were blocked by 20 μM verapamil or 0.1 μM nifedipine.

The effects of angiotensin II on Ca^{2+} fluxes are more controversial. While it is widely believed that this peptide increases ^{45}Ca efflux from preloaded cells, some investigators reported also an enhancement of ^{45}Ca influx (342, 649), whereas others found the opposite (248). According to Kojima et al. (649), the stimulatory effect of angiotensin II on ^{45}Ca influx (as well as the less marked effect of ACTH) was absent at 2 or 10 mM K^+, but potentiated at intermediate K^+ concentrations. Bay K 8644 also increased ^{45}Ca influx at low K^+ concentration, and this effect was additive with those of ACTH or angiotensin II. Increased ^{45}Ca influx in response to K^+ or angiotensin II was blocked by 1 μM nitrendipine, whereas ACTH-induced influx appeared less sensitive to this drug. Using quin-2-loaded cells, Capponi et al. (125) found that angiotensin II evoked a rapid rise in Ca^{2+}_i, which preceded membrane depolarization. Angiotensin II was still able to elicit a rise in Ca^{2+}_i in the presence of verapamil (20 μM) or nifedipine (1 μM). However, these drugs depressed the basal level of Ca^{2+}_i, thereby reducing the concentrations attained under the influence of the stimulator.

Verapamil and D 600 blocked the secretory responses to angiotensin II or K^+ with IC_{50} values of 1 to 10 μM (39, 261, 342, 985). The action of verapamil was noncompetitive with respect to the stimulators, but was antagonized by Ca^{2+} (261). La^{3+} and verapamil appeared less potent on the aldosterone and cyclic AMP responses to ACTH (261). The effect of dihydropyridines on aldosterone secretion by isolated calf glomerulosa cells has been studied by Kojima et al. (650) and Capponi et al. (125). These authors found that these drugs were more effective inhibitors of K^+-induced secretion than angiotensin II-induced secretion. There is evidence that angiotensin II mobilizes an intracellular calcium pool which can be depleted by dantrolene (648), besides enhancing Ca^{2+} entry. After prior treatment with dantrolene, nitrendipine was equally potent on K^+- and angiotensin II-induced secretion (IC_{50} 50 nM). Thus, dihydropyridines might block that part of the angiotensin II-induced secretion attributable to Ca^{2+} influx via voltage-sensitive calcium channels, in agreement with the finding that angiotensin II depolarizes glomerulosa cells (125, 809). Nimodipine (0.1 to 1 μM) strongly reduced ACTH-induced corticosterone secretion in perfused capsular portions of rat adrenal glands (178). However, nifedipine (1 to 10 μM) had little effect on Ca^{2+}-dependent aldosterone secretion stimulated by serotonin (366). [^{3}H]Nitrendipine binding sites with a K_d of 0.3 nM have been described in rat adrenal capsular membranes (284).

In vivo studies also indicated that aldosterone secretion is sensitive to dihydropyridines. Nisoldipine infusion (2.6 μmol/l) into the adrenal arterial supply in sheep blocked aldosterone secretion elicited by elevated K^+ (578). In man, nifedipine diminished the adrenal response to angiotensin II (see ref. 719).

3. Adenohypophysis. The anterior pituitary gland contains several types of secretory cells, which complicates the study of the mechanisms involved in the secretion of individual pituitary hormones. Thus, most investigators have turned to clonal cell lines of tumoral origin, such as GH_3 cells. These cells secrete both growth hormone and prolactin and respond to thyrotropin-releasing hormone (TRH) with an increase in secretion. TRH also enhances the occurrence of action potentials in these cells (see ref. 852).

Secretory cells of adenohypophysis possess voltage-dependent calcium channels, and the existence of two distinct populations of channels in GH_3 cells has been recognized recently (27). Incubation in high-K^+ medium opens calcium channels and triggers secretion. Verapamil and D 600 blocked the effects of K^+-depolarization, though at relatively high concentrations (IC_{50} 10 to 50 μM) in most studies (256, 335, 375, 776, 852, 1051, 1052). However, Enyaert et al. (252) reported an IC_{50} value of 1 μM for verapamil on prolactin release from K^+-depolarized normal and transformed cells. Dihydropyridines were effective inhibitors in normal as well as in transformed cells, and their IC_{50} values ranged from 1 to 17 nM (252, 1051, 1052). Moreover, Enyeart and Hinkle (253) showed that Bay K 8644 increased prolactin secretion from GH_4C_1 cells in a calcium-dependent and nimodipine-sensitive manner. This effect did not require simultaneous treatment with depolarizing concentrations of KCl, probably because GH cells spontaneously fire calcium-dependent action potentials. Ozawa and Kimura (852) reported that D 600 inhibited calcium spikes in GH_3 cells (IC_{50} 30 μM), but also affected sodium and potassium channels at similar concentrations.

TRH stimulation of hormone release is dependent on calcium (for early references, see ref. 376). However, the relative importance of extracellular Ca^{2+} influx *versus* mobilization of intracellular Ca^{2+} has been a matter of controversy. In particular, the use of calcium entry blockers has yielded conflicting results. Some authors found little effect of verapamil or D 600 on TRH-stimulated release of thyroid-stimulating hormone (TSH) (256) or prolactin (375, 776). Others reported inhibitory effects of these drugs (IC_{50} 2 to 50 μM) on TRH-induced TSH release from rat pituitary tissue or TtT cells (335, 375, 970), or on TRH-induced prolactin secretion by GH_3 cells (852, 1052). Flunarizine (1 μM) was found to block TRH-induced prolactin secretion without affecting high-K^+-induced secretion (744). Nifedipine (0.3 to 3 μM) attenuated TRH-stimulated prolactin release in rat adenohypophysis (1045). According to Enyeart et al. (252), nisoldipine was much less active on TRH-induced prolactin secretion than on high-K^+-evoked secretion.

Tan and Tashjian (1052) showed that TRH-stimulated prolactin release from GH_4C_1 cells could be resolved into two components, the major component being dependent on calcium entry and inhibited by verapamil or nifedipine. The IC_{50} of nifedipine was estimated to be 60 nM, as compared to 20 nM for high-K^+-stimulated ^{45}Ca uptake or prolactin release. Using quin-2, Albert and Tashjian (11) were able to follow the early time course of cytosolic free Ca^{2+} concentration in GH cells after stimulation by TRH. They noted a "spike phase" to micromolar levels which decayed to a near basal concentration, followed by a "plateau phase" of elevated $Ca^{2+}{}_i$. The early spike phase reflects the mobilization of an intracellular calcium pool, which is thought to follow activation of phosphatidylinositol 4,5-bisphosphate breakdown (377). The temporal pattern of TRH-induced prolactin secretion could also be resolved into two similar phases. In contrast, using aequorin or quin-2, other investigators found only the early spike phase of elevated $Ca^{2+}{}_i$ after TRH stimulation (378, 1000). Albert and Tashjian (11) reported that nifedipine (2.5 μM) or verapamil (100 μM) had little effect on the spike phase, but inhibited by about 60% the plateau phase of the rise in $Ca^{2+}{}_i$ and hormone release. Similar results have been recently reported by Gershengorn and Thaw (379).

Fewer data are available on gonadotropin release. Nifedipine depressed lactogenic hormone (LH) release stimulated by lactogenic hormone-releasing hormone (LHRH) in rat adenohypophysis (1045). Cultured gonadotrophs from ovine adenohypophysis showed increased membrane voltage fluctuations (without action potentials) in response to gonadotropin-releasing hormone (GnRH), and this effect of GnRH was depressed by 6 μM nifedipine (727). However, in rat pituitary cells, gonadotropin release by GnRH was not influenced by nifedipine and other dihydropyridines, whereas it was inhibited by verapamil or D 600 (48, 171, 807).

Usually, basal release of pituitary hormones was found to be little affected by calcium entry blockers. However, basal prolactin release from rat pituitary cells was depressed by flunarizine (IC_{50} 0.15 μM), and this inhibition was antagonized by Ca^{2+} (206). Takahara et al. (1045) found that nifedipine (3 μM) inhibited basal prolactin [but not growth hormone (GH) or LH] release from rat adenohypophysis, whereas Tan and Tashjian (1052) found no effect of nifedipine on basal prolactin release from GH_4C_1 cells. Albert and Tashjian (11) reported that nifedipine (2.5 μM) produced a decrease in the rather high basal cytosolic Ca^{2+} concentration (300 nM) in GH cells.

In man, verapamil was able to depress the gonadotropin response to GnRH or TRH, and the TSH (but not the prolactin) response to TRH (41, 42, 1116). In contrast, neither nifedipine (1032) nor diltiazem (1116) inhibited the in vivo release of pituitary hormones.

4. Endocrine pancreas: beta-cells. Glucose is the primary stimulus of insulin release, which is also controlled by cholinergic and adrenergic nerves. Insulin secretion is caused by a cytosolic accumulation of Ca^{2+}, and the Ca^{2+}-dependent insulin release is modulated by cyclic AMP (1169). The correlation between the rise in the concentration of cytosolic Ca^{2+} (monitored by the quin-2 method) and insulin release has been demonstrated in an insulin-secreting cell line by Wollheim and Pozzan (1168). Glucose and tolbutamide depolarize the beta-cell membrane and induce bursts of spike activity that are blocked by 50 μM D 600 and restored by elevating extracellular Ca^{2+} (501, 731). Cyclic AMP may act, at least partly, by increasing the permeability of calcium channels in the plasma membrane of beta-cells (502).

D 600 and verapamil inhibit glucose- and sulfonylurea-induced insulin release (214, 677, 707). The IC_{50} of verapamil on glucose-induced secretion was 3 μM when the perfusion medium contained 2 mM Ca^{2+}, but less than 0.8 μM in the presence of 0.6 mM Ca^{2+} (214). The inhibitory effect of verapamil could be related to depression of calcium influx through voltage-dependent calcium channels, which are opened following glucose-induced depolarization (708). In contrast, verapamil or D 600 did not affect the decreased rate of ^{45}Ca efflux elicited by glucose in the absence of extracellular Ca^{2+}, which might be due to inhibition of Na^+-Ca^{2+} exchange (509).

Nifedipine was more potent than verapamil at antagonizing glucose-evoked insulin release from rat islets (IC_{50} 80 nM) (706), whereas diltiazem was somewhat less potent (IC_{50} 10 μM) (1173). Bay K 8644 (0.2 to 20 μM) and CGP 28392 enhanced glucose-stimulated calcium uptake and insulin release, and protected the beta-cell against the inhibitory action of nifedipine (709, 710).

Insulin secretion in response to a constant glucose stimulus is biphasic, consisting of a first "spike" phase, followed by a sustained period of release (1169). The spike phase was unaffected by 5 μM verapamil when this drug was administered concomitantly with the glucose stimulus. Since verapamil blocked glucose-stimulated calcium uptake during the second phase of secretion, Wollheim and Sharp (1169) proposed that this early phase was not triggered by enhanced Ca^{2+} inflow, but rather by a mobilization of calcium stores. Inositol trisphosphate might be the second messenger releasing Ca^{2+} from endoplasmic reticulum in insulin-secreting cells (589, 883). The biphasic pattern of insulin release has medical implications, since patients with certain types of diabetes lack only the initial response to glucose (see ref. 1169).

Lebrun et al. (669) noted that insulin secretion evoked by K^+-depolarization was more sensitive to verapamil than was glucose-stimulated release, especially when the glucose concentration was suboptimal. From their study of ^{45}Ca fluxes, they concluded that glucose at low concentrations might stimulate Ca^{2+} inflow by gating voltage-insensitive channels. In insulin-secreting clonal cells, verapamil or diltiazem (20 μM) almost abolished the effects of high-K^+ and alanine on $Ca^{2+}{}_i$ and insulin

release, but reduced the responses to glyceraldehyde by only 50% (1168).

Acetylcholine stimulates insulin release via muscarinic receptors, and its stimulatory effect is largely mediated by an influx of Ca^{2+}, as shown in particular by the use of verapamil (1170).

In intact animals, diltiazem, at vasoactive doses, did not interfere with basal or glucose-induced insulin secretion (1174). In rats, nifedipine dose-dependently reduced glucose tolerance and insulin release after i.v. glucose (598). In man, verapamil has been shown to inhibit stimulated insulin secretion (204). However, variable results have been reported with nifedipine, and, on the whole, it appears that calcium antagonists do not affect glucose homeostasis to a clinically relevant degree (for a review, see ref. 1074).

5. Kidney: juxtaglomerular cells. Renin secretion by juxtaglomerular cells, as part of the renin-angiotensin-aldosterone system, plays a crucial role in electrolyte homeostasis and in the modulation of arterial blood pressure. The various factors affecting renin secretion in vivo seem to act via two second messengers, Ca^{2+} and cyclic AMP (for a review, see ref. 160). Beta-adrenoceptor agonists stimulate renin release by enhancing the synthesis of cyclic AMP. When Ca^{2+} acts as a second messenger, it appears to inhibit renin secretion, in contrast with the situation prevailing in most other endocrine systems.

High-K^+ inhibits renin secretion from rat renal cortical slices by a calcium-dependent mechanism, and this effect was antagonized by D 600, verapamil, or diltiazem (159, 162, 348, 855). IC_{50} values were 0.1, 0.5, and 2 μM, respectively. The effects of calcium entry blockers were increased when external Ca^{2+} was lowered. Thus, it is likely that depolarization opens voltage-sensitive calcium channels and increases Ca^{2+} influx in juxtaglomerular cells, thereby inhibiting renin release. A similar mechanism would explain the influence of vascular stretch in the vicinity of juxtaglomerular cells on renin secretion. Indeed, inhibition of secretion by increased stretch is associated with depolarization of juxtaglomerular cells (349), and this inhibition was blocked by verapamil (348).

Ouabain and low extracellular K^+ inhibit renin secretion. According to Churchill et al. (158), D 600 did not interfere with the effects of ouabain or low K^+, in agreement with their likely mechanism of action, i.e., inhibition of the sodium pump, leading to inhibition of Ca^{2+} extrusion through Na^+-Ca^{2+} exchange. However, in other studies, verapamil (5 μM) was able to block the inhibitory effect of ouabain (182, 855).

Angiotensin and vasopressin inhibit renin secretion, and their effects are blocked by Ca^{2+} chelators. Again, controversial results have been obtained with verapamil and D 600 (160). Alpha-adrenoceptors mediate inhibition of renin secretion by a mechanism that is sensitive to these drugs (693, 845). In contrast, adenosine inhibition of renin secretion was unaffected by D 600 (161).

Neither D 600 (3 μM) nor diltiazem (30 μM) affected basal renin release from rat cortical slices (160, 162). However, nifedipine (1 to 10 μM) markedly enhanced renin secretion (26). In the presence of a moderately depolarizing concentration of K^+, Bay K 8644 (10 μM) inhibited renin secretion, whereas it had the opposite effect in normal Krebs buffer (733).

When infused into the renal arteries of anesthetized dogs, verapamil and nifedipine stimulated renin release, but this effect was observed only when systemic hypotension occurred (1). In isolated rat kidney perfused at constant pressure, renin release was stimulated about 3-fold by 1 μM nitrendipine (695). This model circumvents the indirect actions of vasodilating agents. In anesthetized rats, D 600 decreased renin levels (113). This unexpected result may arise from divergent actions on several parameters that control renin secretion. Indeed, calcium entry blockers induce natriuresis, which could depress renin secretion through the macula densa mechanism.

In man, acute, but not chronic, administration of diltiazem, nifedipine, and nitrendipine has been reported to increase plasma renin activity (for references, see ref. 695). However, nifedipine did not counteract the secretion of renin evoked by angiotensin II (756).

6. Testis and ovary. a. LEYDIG CELLS. Testosterone production by these cells can be stimulated by LH and GnRH. The action of LH is mediated principally by cyclic AMP, but in the absence of calcium, or in the presence of verapamil, testosterone production in reponse to LH is decreased (571, 687). The stimulatory effect of GnRH is calcium dependent and can be blocked by nifedipine (685, 1034). Nifedipine (30 nM) also blocked the testosterone production evoked by a phorbol ester that activates protein kinase C (686).

b. GRANULOSA CELLS. Both cyclic AMP and Ca^{2+} are involved in the regulation of ovarian steroidogenesis by gonadotropins. Verapamil (125 μM) did not influence cyclic AMP production by rat granulosa cells in response to follicle-stimulating hormone (FSH), but reduced progesterone production (1076, 1077). Ca^{2+} is also required for stimulation by LH of pregnenolone biosynthesis from endogenous sterol in swine ovarian cells (1117).

7. Parathyroid and thyroid. a. PARATHYROID. Parathormone release is inversely related to the extracellular concentrations of Ca^{2+} and Mg^{2+} (115). Direct measurement of intracellular Ca^{2+} by the quin-2 method has revealed that the cytosolic Ca^{2+} concentration increases when the extracellular Ca^{2+} concentration is raised from 1 to 2 mM (987). Increasing the extracellular Ca^{2+} concentration depolarizes parathyroid cells (117), and this effect could account for the unusual relationship between the concentrations of extra- and intracellular Ca^{2+} in this cell type. However, the effects of calcium entry blockers on parathormone secretion have not been studied.

b. THYROID. The action of TSH on thyroid cells is

essentially mediated by cyclic AMP (231). However, cytosolic Ca^{2+} also plays a regulatory role. Agents that increase Ca^{2+}_i (high-K^+, calcium ionophores) mimic some effects of TSH (protein iodination, glucose oxidation), but reduce TSH-evoked secretion and cyclic AMP accumulation. The effects of calcium entry blockers on the secretion of thyroid hormones have not been investigated.

F. Circulating Cells and Mast Cells

1. Platelets. Platelets respond to a variety of agents such as ADP, collagen, thrombin, and 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor, PAF-acether) by changing shape, producing thromboxane A_2, releasing granule contents (adenine nucleotides, serotonin), and aggregating. The platelet response thus involves both contractile and secretory events. Platelet activation is antagonized by agents that increase cyclic AMP, for instance prostacyclin. Inhibition of platelet cyclooxygenase prevents secretion and secondary aggregation in response to some stimuli such as ADP, but not shape change or primary aggregation (916).

A rise in cytosolic Ca^{2+} has been regarded as the common determinant of the various processes involved in platelet activation, and the secretory response to several excitatory agonists, including PAF-acether, is closely associated with Ca^{2+} uptake (163, 674). But this rise might be accounted for, at least partly, by a mobilization of internal stores, since platelet activation can be evoked by calcium ionophores even in the absence of external Ca^{2+} (269). Also, intracellular release of membrane-bound Ca^{2+} (measured by chlortetracycline fluorescence) precedes platelet shape change and secretion evoked by thrombin or collagen (270, 483). More recently, direct measurements using quin-2 have confirmed that an increase in Ca^{2+}_i occurs on stimulation by ionomycin (a calcium ionophore), thrombin, ADP, and PAF-acether (479, 480, 918). The rise in Ca^{2+}_i was markedly reduced on removal of extracellular Ca^{2+}, which suggests that this rise was mainly due to influx of external Ca^{2+}. Aspirin only slightly reduced the Ca^{2+}_i changes evoked by thrombin, PAF-acether, and ADP, indicating that thromboxane A_2 is not a major mediator of the Ca^{2+} movements (479, 480).

Although Ca^{2+} is obviously an important mediator of platelet activation, thrombin and PAF-acether, but not ionomycin, could partly activate calcium-depleted platelets without a concomitant rise in Ca^{2+}_i (480, 918). Rink et al. (917) showed that diacylglycerol and phorbol ester stimulate platelet secretion without raising Ca^{2+}_i, probably by activating protein kinase C. Excitatory agonists such as thrombin, collagen, and PAF-acether stimulate phosphoinositide breakdown, thereby producing diacylglycerol and inositol 1,4,5-trisphosphate (7, 1131). Diacylglycerol, acting via protein kinase C, and inositol 1,4,5-trisphosphate, which mobilizes platelet calcium stores (850) and thereby activates a Ca^{2+}-calmodulin-dependent kinase, seem to act synergistically to induce the platelet response (for a review, see ref. 828).

Calcium entry blockers were found to inhibit platelet aggregation and serotonin release evoked by various agents (483, 548, 739, 843, 844), but inhibitory effects required rather high concentrations. For instance, IC_{50} values for inhibition of platelet aggregation and thromboxane production induced by ADP and thrombin were higher than 100 μM for diltiazem, verapamil, nifedipine, and nimodipine in the study of Onoda et al. (844). With diltiazem, there was no marked difference in antiaggregatory potency between *d*- and *l*-stereoisomers, in contrast with inhibition of smooth muscle contraction (640). At concentrations effective against vascular contraction, flunarizine did not inhibit serotonin release or thromboxane synthesis in thrombin-stimulated platelets (196).

Platelet activation by PAF-acether was relatively sensitive to chelation of extracellular Ca^{2+} and to phenylalkylamines (IC_{50} 10 to 20 μM) and diltiazem (IC_{50} 13 μM) (167), but was resistant to nicardipine (703). At a concentration of 2 μM, verapamil did not inhibit platelet aggregation induced by PAF-acether, but caused previously aggregated platelets to desaggregate (630). The effects of verapamil, D 600 and diltiazem on platelet activation by PAF-acether could reflect interference with the binding of PAF-acether to its receptor (480, 1080). Similarly, when platelets are stimulated by epinephrine, verapamil (but not nifedipine or nisoldipine) inhibits platelet responses by acting as a competitive antagonist at the level of the platelet alpha$_2$-adrenoceptor (43, 587, 780). Phenylalkylamines also interfere with serotonin binding at platelet S_2 receptors (6).

According to Erne et al. (255), the agonist dihydropyridine, CGP 28392, (1 μM) caused an increase in Ca^{2+}_i from 120 to 200 nM in human platelets, which was abolished in the presence of 3 mM EDTA. This stimulatory effect was antagonized by nitrendipine (IC_{50} 6 nM), but not by verapamil or diltiazem (up to 10 μM). In sharp contrast, other investigators reported that another calcium agonist, Bay K 8644, did not affect Ca^{2+}_i in human platelets (226). These authors questioned the existence of voltage-sensitive calcium channels in platelets, since high potassium did not increase Ca^{2+}_i (see also ref. 479). In addition, they showed that the resting Ca^{2+}_i level and the thrombin-induced rise in Ca^{2+}_i were unaffected by verapamil, diltiazem, or PN 200-110 (up to 10 μM). Thus, the calcium influx induced by thrombin seems to occur via a pathway that is not a voltage-sensitive calcium channel. Similarly, ADP (479) and PAF-acether (703) could open receptor-operated calcium channels that are resistant to dihydropyridines. The reason why CGP 28392, but not Bay K 8644, induced a nitrendipine-sensitive increase in Ca^{2+}_i in platelets remains to be elucidated. Specific [^{3}H]nitrendipine binding sites could not be detected in human platelets (388, 780).

Recently, Resink et al. (903) found that CGP 28392 induced platelet activation (shape change, protein phos-

phorylation) independently of the presence of external Ca^{2+}. This shape change required higher CGP 28392 concentrations than did the rise in Ca^{2+}_i [50% effective concentration (EC_{50}) 6 μM *versus* 0.2 μM] and was unaffected by 0.1 μM nitrendipine. The authors suggested that CGP 28392 at comparatively high concentrations could evoke a mobilization of intracellular calcium stores. Moreover, part of the effects of calcium entry blockers, especially at high concentration, might be due to an interference with activation of protein kinase C. Such an action has been described for verapamil and other drugs interacting with phospholipids (775).

Though, at plasma levels that are achieved in vivo, calcium entry blockers do not affect platelet activation in vitro, nifedipine and nisoldipine, when administered to humans, have been shown to decrease ADP- and collagen-induced aggregation of platelets ex vivo and to increase the bleeding time (187, 584, 972). In a model of thrombosis in dogs, verapamil and nifedipine, at concentrations that are encountered in clinical practice, proved to be platelet inhibitors as effective as dipyridamole (886). In another study, however, no significant effect on ADP-induced aggregation was observed after 4 days of treatment with verapamil or nisoldipine (587). Diltiazem did not influence the bleeding time, but decreased the aggregation induced by threshold concentrations of ADP or epinephrine (181). In vivo inhibitory effects of calcium entry blockers might be explained by a synergism with those of other antiaggregatory agents such as prostacyclin (548, 844). Indeed, verapamil and diltiazem have been shown to increase the production of prostacyclin by vascular tissue (see ref. 738).

2. *Basophils and mast cells.* The release of histamine and other mediators from mast cells and basophil granulocytes is considered the main mechanism of the immediate-type allergic reactions. These cells bear specific receptors that bind immunoglobulin E (IgE) upon sensitization. Bridging of cell-bound IgE by multivalent antigen, or by IgE-directed ligands such as concanavalin A, induces increased methylation of phospholipids, followed by Ca^{2+} influx, slow-reacting substance (SRS) synthesis and release, and degranulation (555, 556). An increase in Ca^{2+}_i following mast cell activation has been demonstrated by White et al. (1153). Some increase in Ca^{2+}_i also occurred in the absence of external calcium. IgE-directed ligands induce a phosphatidylinositol response in mast cells (for a review, see ref. 449).

The importance of Ca^{2+} for histamine release from mast cells is indicated by the stimulatory effect of calcium ionophores, which is abolished in calcium-depleted cells (339). The antiallergic drug, cromoglycate (cromolyn), inhibits Ca^{2+} influx and degranulation mediated by IgE receptors (662), but not the effects of A 23187 (340). Cromoglycate may act by binding to a specific membrane protein, which is likely to be a constituent of the putative channel operated by IgE receptors (734). Alternatively, antiallergic drugs may exert an ill-defined, general stabilizing effect on the mast cell membrane (864).

Mast cells appear to be devoid of voltage-dependent calcium channels (934). Usually, calcium entry blockers showed inhibitory effects on mast cells activation only at high concentrations (46, 145, 251, 633, 676, 752, 919, 1039, 1053). For instance, in the study of Middleton et al. (752), verapamil or D 600 (10 to 50 μM) and nifedipine (1 μM) did not inhibit antigen-induced histamine release from human basophils. However, Chand et al. (146) reported an IC_{50} value of 3.7 μM for verapamil on human basophils. Nifedipine and verapamil were more potent on SRS production (nifedipine IC_{50} 1 μM) than on histamine release induced by antigen or anti-IgE antibodies in human lung (142, 675). In rat basophil leukemia cells, SRS synthesis evoked by A 23187 was also inhibited by verapamil and nifedipine (IC_{50} 1.8 and 7.5 μM, respectively) (683). Several studies reported that calcium entry blockers showed similar inhibitory potencies on histamine release evoked by antigen and A 23187, which indicates that their action lacks specificity (145, 251, 676, 856, 1053). Incubation of ovalbumin-sensitized guinea pig lung tissue in high-potassium medium did not markedly stimulate the release of SRS, and this release was unaffected by 10 μM verapamil (188). On the whole, it seems unlikely that the beneficial effects of nifedipine and verapamil in exercise-induced asthma (see section III B 2) might be attributed to their effects on mast cells (676, 919).

3. *Neutrophils, lymphocytes, and erythrocytes.* a. NEUTROPHILS. Calcium plays an important regulatory role in neutrophil functions, including stimulation of locomotion by chemotactic factors, lysosomal enzyme secretion, and superoxide anion generation, as shown initially by manipulation of the external Ca^{2+} concentration and by the use of ionophores (991). The chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP), increases the membrane permeability to Ca^{2+}, especially in the presence of cytochalasin B, and this increase is correlated with enhanced lysosomal enzyme release (96, 797). The Ca^{2+}_i rise evoked by FMLP precedes the onset of superoxide production (653, 882). However, chelation of external Ca^{2+} with EGTA did not abolish the Ca^{2+}_i rise and subsequent activation induced by various stimuli, indicating that enhanced Ca^{2+} influx is not an absolute requirement for neutrophil activation (653, 882, 999). Although important, the Ca^{2+}_i rise is not sufficient for cell activation, since FMLP at low concentration or ionomycin elicited a maximal increase of Ca^{2+}_i without concomitant degranulation or superoxide generation (653, 882, 1154). Moreover, phorbol myristate acetate activated neutrophils without eliciting an increase in Ca^{2+}_i. Thus, it is likely that receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate is implicated in neutrophil activation by FMLP (1178).

Verapamil (30 to 300 μM) inhibited superoxide gener-

ation and ^{45}Ca uptake evoked by FMLP (but not the binding of FMLP to its receptor), and this effect was overcome by increasing the external Ca^{2+} concentration (991). High concentrations of verapamil (> 100 μM) also depressed lysosomal enzyme release evoked by FMLP or A 23187 (246). Nifedipine depressed the release of PAF-acether, SRS, and beta-glucuronidase, as well as ^{45}Ca uptake, elicited by A 23187 or zymosan (590). SRS production evoked by A 23187 was more sensitive to nifedipine (IC_{50} 0.1 μM) than was PAF-acether synthesis (IC_{50} 1 μM) or lysosomal enzyme release (IC_{50} > 5 μM). The inhibition of PAF-acether synthesis was reversed by increasing the extracellular Ca^{2+} concentration. Human neutrophils treated with 1 μM verapamil or nifedipine exhibited a reduced capacity to kill bacteria (618). Calcium antagonists (verapamil, D 600, diltiazem, prenylamine, perhexiline) inhibited chemotaxis at relatively high concentrations (247). Calcium channels are probably not involved in this effect, since inhibition was more pronounced in the absence of external Ca^{2+}. Moreover, inorganic Ca^{2+} antagonists did not inhibit chemotaxis.

b. LYMPHOCYTES. Ca^{2+} is probably an important second messenger in the action of lymphocyte mitogens (for references, see ref. 464). Using quin-2, Tsien et al. (1079) showed that T-cell mitogens cause an early rise of Ca^{2+}_i. The hydrolysis of phosphatidylinositol 4,5-bisphosphate may also be a key event in the action of mitogenic agents (67).

Lymphocyte proliferation is antagonized by calcium entry blockers (75, 77, 464, 628). Half-maximal inhibition of [^{3}H]thymidine incorporation elicited by stimulation with lectins was observed at a concentration of 10 to 20 μM for verapamil, nifedipine, and nicardipine, and 80 μM for diltiazem. Inhibition was not reversed by increasing the external Ca^{2+} concentration. Verapamil also inhibited the ^{45}Ca influx evoked by phytohemagglutinin (75).

c. ERYTHROCYTES. Erythrocyte deformability is an important determinant of blood viscosity, which may be increased in peripheral arterial diseases (897). Flunarizine decreases blood hyperviscosity by improving red cell deformability (197). This effect might be mediated by a depression of the calcium permeability of the red cell membrane, since intracellular calcium loading reduces the ability of red cells to deform. Indeed, flunarizine (5 to 20 μM) inhibited the shape changes (crenation) of human red cells in which the Ca^{2+} influx was enhanced by A 23187 (194). In this study, flunarizine was more potent than cinnarizine and lidoflazine, and verapamil and D 600 had little effect at a concentration of 10 μM. Diltiazem (at least 100 μM) also improved red cell deformability, but this effect was shared by the (pharmacologically inactive) *l-cis*-isomer and by propranolol (955). Nifedipine has been shown to improve red cell deformability after sublingual administration (10 mg) to patients (1124). However, nifedipine, nitrendipine, and nisoldipine did not influence red cell filterability when cells from healthy volunteers were filtered within 2 h of venopuncture (1007). In contrast, nifedipine and nitrendipine significantly improved the filterability of stored red blood cells. These effects of calcium entry blockers on red cell deformability are probably linked to a membrane-stabilizing action of these drugs and mediated by interactions with membrane phospholipids (955).

Calcium entry blockers also interfere with nucleoside transport in red cells. Ford et al. (338) reported that verapamil and diltiazem competitively inhibited adenosine transport (K_i 14 and 160 μM, respectively). Striessnig et al. (1029) found that (+)nimodipine was a potent competitive inhibitor (IC_{50} 0.6 μM with 1 μM adenosine), and that (−)nimodipine and other dihydropyridines were less active. In parallel binding studies, these authors showed that red cells possessed dihydropyridine binding sites with inverse stereoselectivity with respect to calcium channel receptor sites present in other tissues (see section III A). That this particular binding site could be identified as the nucleoside transporter was confirmed by the finding that nucleosides and nucleoside uptake inhibitors blocked [^{3}H]nimodipine binding in erythrocytes.

G. Miscellaneous Tissues

1. Skeletal muscle. Skeletal muscle contraction is produced by the release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum (SR) in response to depolarization of the transverse tubule membrane. The exact mechanism by which this depolarization is translated into Ca^{2+} efflux from the SR is still debated (344, 964). One of the current hypotheses proposes that inositol trisphosphate is released by the T-tubular membrane when this membrane is depolarized, and that this chemical messenger triggers the efflux of Ca^{2+} from the SR (1120).

Electrophysiological studies, conducted mainly on amphibian muscle, have revealed the existence of voltage-dependent calcium channels in skeletal muscle (for references, see ref. 629). These channels resemble those found in other tissues, but they react to membrane potential changes one or two orders of magnitude more slowly. Skeletal muscle calcium channels appear to be located predominantly in transverse tubules (16, 824). Slow (calcium) action potentials can be elicited in Cl^--free solutions, and they are sensitive to verapamil and bepridil, their amplitude being half-maximally depressed at a concentration of about 30 nM (629). In voltage-clamp experiments, the channels were found to be blocked by nifedipine (IC_{50} 0.2 to 0.9 μM) (17, 549), PN 200-110 (IC_{50} 0.4 μM) (976), diltiazem (IC_{50} 80 μM) (17), and D 600 (IC_{50} 14 μM) (735). Ildefonse et al. (549) reported that Bay K 8644 (0.1 μM) increased I_{Ca}.

The role played by the voltage-dependent calcium channels of T-tubules in excitation-contraction coupling remains a matter of speculation. When external Ca^{2+} is

lowered to 10 nM, the fibers can produce normal twitches for many minutes (702). By observing contraction under voltage-clamp, McCleskey (735) found that nifedipine or D 600, at concentrations more than sufficient to block calcium channels, did not impair contraction, whether administered extra- or intracellularly. However, by recording simultaneously the calcium current and tension from single fibers, Ildefonse et al. (549) showed that a component of the contraction was associated with I_{Ca} and was depressed by nifedipine and enhanced by Bay K 8644. Calcium influx might be more important for maintained contraction, such as tetanus or K^+ contracture. Indeed, K^+ contractures were rapidly inhibited by lowering external Ca^{2+} (345, 1022), and they were depressed by low concentrations of calcium entry blockers (346, 956). Ca^{2+} entry through T-tubule channels may also increase the Ca^{2+} load of the SR and, thus, the Ca^{2+} available for release upon depolarization. This might contribute to the phenomenon of posttetanic potentiation of twitch contraction (824).

Although Ca^{2+} influx seems to be of little importance for twitch contraction, calcium entry blockers, when used at relatively high concentrations, may affect twitch contraction. Both potentiation and depression effects have been reported, sometimes in succession. For instance, verapamil (5 μM) evoked a long-lasting increase in twitch contraction of rat isolated diaphragm, which was followed by depression of contraction (50% after 53 min), and addition of Ca^{2+} failed to antagonize the blocking action of verapamil (1113). Similar biphasic effects of verapamil and D 600 have been noted with frog skeletal muscle (346, 724). In other studies, only facilitatory (218, 994) or inhibitory (IC_{50} 26 μM) (72, 89) effects of verapamil or D 600 have been observed. Gallant and Goettl (363) compared the action of several calcium entry blockers at a concentration of 25 μM on several isolated muscles from rodents. They found inhibitory effects with verapamil, D 600, bepridil, and diltiazem on twitch and/or tetanic tension, especially on murine muscles, whereas nitrendipine and lidoflazine had no effect, and nifedipine potentiated twitch contraction. Verapamil and (+) D 600 induced a marked fading of the tetanus plateau. The effects of verapamil were not antagonized by raising the Ca^{2+} concentration. At low concentrations, diltiazem has been found to potentiate twitch contraction (450, 1125). In mouse muscle, the maximal effect (40 to 45% increase in tension) was obtained with 50 to 100 nM *d-cis*-diltiazem, but the *l-cis* isomer was less potent (1125).

The mechanisms of these facilitatory and inhibitory effects of calcium entry blockers are not clear. Walsh et al. (1125) showed that low concentrations (0.5 to 1 μM) of diltiazem decreased the mechanical threshold in rodent muscle. They suggested that diltiazem may potentiate twitch contraction by stimulating SR calcium release through its specific binding site in the T-tubular membrane. Dörrscheidt-Käfer (218) found that D 600, at concentrations of 20 μM and higher, facilitated contraction by shifting the mechanical threshold to more negative potentials, and that the (+)isomer, which is more active as a local anesthetic agent, was more potent than the (−)isomer. Skirboll et al. (994) reported that the potentiating effect of verapamil on twitch responses was accompanied by an increase in the duration of the action potential, possibly by an action on a K^+ conductance. At high concentrations, verapamil and D 600 may depress contraction by blocking Na^+ channels (89, 218). These drugs elicited a significant decrease of the resting membrane potential at concentrations of 25 μM or higher (218, 363). Direct effects on calcium release from the SR appear unlikely. Indeed, high concentrations (>100 μM) of nifedipine or D 600 did not impair contraction after intracellular administration (735). Verapamil (25 μM) did not affect caffeine contractures in murine muscle (363). In contrast, verapamil, diltiazem, and nifedipine antagonized the contracture elicited by acetylcholine (155, 957).

Some authors reported that contractions elicited by nerve stimulation were more sensitive to calcium entry blockers than were contractions evoked by direct stimulation (32, 668, 957). Nevertheless, the high concentrations that were usually needed to interfere with skeletal muscle contraction are not likely to be encountered under clinical circumstances. However, verapamil and nifedipine have been reported to potentiate the effect of neuromuscular blocking agents, both in vitro and in vivo (73).

2. Exocrine tissues. a. EXOCRINE PANCREAS. Pancreatic enzyme secretion is controlled primarily by the gut hormone cholecystokinin and by acetylcholine, and the action of these secretagogues involves a rise of $Ca^{2+}{}_i$ (1160). This rise occurs mainly by mobilization of intracellular calcium stores, since deprivation of extracellular Ca^{2+} does not impair enzyme release, at least during the initial 10 min of incubation with secretagogue (369, 1160). According to the present view, agonists such as carbachol stimulate polyphosphoinositide breakdown (887), thereby generating inositol trisphosphate and diacylglycerol. Inositol trisphosphate mobilizes intracellular calcium stores (1028), and the resulting transient rise in $Ca^{2+}{}_i$ would be responsible for the initial, rapid phase of pancreatic enzyme secretion; the subsequent, sustained phase of secretion would be related to activation of protein kinase C by diacylglycerol (745, 854). The $Ca^{2+}{}_i$ rise elicited by carbachol (1 μM) was similar in the presence or absence of external Ca^{2+}, but the return to resting levels was distinctly more rapid in the absence of external Ca^{2+} (838).

Pancreatic acinar cells do not appear to have voltage-dependent calcium channels (871). At a concentration of 100 μM, D 600 had no effect on ^{45}Ca fluxes in mouse pancreatic acini in the presence or absence of a cholinergic agent (217). Amylase release from rat acini in response to carbachol (1 μM) was little inhibited (20%) by 10 μM verapamil or diltiazem, but was more sensitive

to the putative intracellular calcium antagonist TMB-8 (547). In man, at the highest dose recommended in cardiology, verapamil did not modify the pancreatic secretory responses evoked by cholecystokinin, secretin, or sham feeding (825).

b. GASTRIC MUCOSA. Parietal cell acid secretion is regulated by at least three different types of secretagogue, histamine, acetylcholine, and gastrin. Histamine acts through H_2 receptors and increases cyclic AMP in parietal cells. In contrast, the effects of acetylcholine and gastrin are dependent on extracellular Ca^{2+} (for references, see ref. 154). Carbachol caused a sustained increase in $Ca^{2+}{}_i$ in dog isolated parietal cells (784).

In the guinea pig isolated gastric fundic mucosa, verapamil inhibited acid secretion evoked by histamine (IC_{50} 100 μM), but did not affect acid secretion in response to theophylline or theophylline plus dibutyryl cyclic AMP (636). In contrast, in guinea pig isolated parietal cells, verapamil and D 600 blocked with identical potency (IC_{50} 10 to 20 μM) acid secretion stimulated by histamine, high potassium, and dibutyryl cyclic AMP (980). In view of this apparent nonselectivity, these authors suggested that verapamil and D 600 interfered with the parietal cell proton pump [$(H^+ + K^+)$ATPase]. Indeed, verapamil and other tertiary amines have been shown to inhibit this enzyme by competing with K^+ (550). In rabbit isolated gastric glands, verapamil and nicardipine inhibited acid secretion in response to carbachol and histamine, but their potencies were similar in the absence or presence of external Ca^{2+} (154). With verapamil, the IC_{50} values were 2 to 7 μM. Nifedipine (10 μM) did not modify acid secretion. Thus, Chew (154) concluded that the inhibitory effects of verapamil and nicardipine were nonspecific. In man, verapamil depressed the acid response to gastrin (636, 1006).

3. Other tissues. a. KIDNEY TUBULES. Several studies indicate that calcium entry blockers (e.g., nifedipine, nitrendipine, felodipine, verapamil, D 600, and diltiazem) inhibit renal tubular water and electrolyte reabsorption (for references, see refs. 216, 371, 621, and 1186). When doses that caused a comparable fall in blood pressure were administered to hypertensive patients, nifedipine markedly increased water and sodium excretion, whereas verapamil and diltiazem had lesser effects on natriuresis (1186). These effects do not appear to be caused by a modification of the glomerular filtration rate or of renin secretion.

b. LIVER. In hepatocytes, stimulation by $alpha_1$-adrenoceptor agonists and vasoactive peptides promotes the activation of phosphorylase and glycogen breakdown by a Ca^{2+}-dependent mechanism. According to Joseph et al. (588), entry of extracellular Ca^{2+} into the cell is required to obtain a sustained hormonal stimulation of phosphorylase activity. Rather high concentrations of diltiazem are needed to block extracellular Ca^{2+}-dependent effects (IC_{50} 120 μM).

c. SPERM. Diltiazem, flunarizine, and verapamil have been shown to stimulate human sperm motility in vitro (537). Similar results were obtained with Ca^{2+} chelators.

III. Mechanisms of Action of Calcium Entry Blockers

As mentioned above (section II A), the first indication of the presence of Ca^{2+} channels in pericellular membranes was found in 1953 by Fatt and Katz (267), who studied action potentials in crustacean muscle fibers. Electrophysiological studies of cardiac preparations allowed Reuter (905) and Coraboeuf and coworkers (931) to extend this concept by the description of the slow inward current (section II C). In smooth muscle, Bolton (87) and Van Breemen et al. (1092) have proposed that POCs could be different from ROCs. In this section, we will examine the interaction of calcium entry blockers with calcium channels, as revealed by binding and electrophysiological studies. Additional possible sites of action will then be considered, mainly calmodulin and alpha-adrenoceptors. Finally, possible mechanisms accounting for the selectivity of calcium entry blockers will be discussed.

A. Specific Binding Sites

Since [^{3}H]nitrendipine became available a few years ago (63), a new research area, dealing with specific binding sites, has developed in the field of calcium antagonism. A series of new labelled ligands is now commercially available that includes (+)-[^{3}H]desmethoxyverapamil (278), *d-cis*-[^{3}H]diltiazem (392), (+)-[^{3}H]PN 200-110 (see table 8), [^{3}H]PY 108-068 (1035), [^{3}H]Bay K 8644 (61), [^{125}I]iodipine (276), and reagents for affinity labelling, [^{3}H]dihydropyridine isothiocyanate (1118) and [^{3}H]azidopine (281).

1. General features and effects of cations. a. DIHYDROPYRIDINE BINDING. In all tissues examined so far, including human brain (870) and heart (277), binding studies have generally revealed a single class of sites that bind dihydropyridines reversibly and with high affinity. In most tissues the dissociation constant (K_d) for [^{3}H]nitrendipine or [^{3}H]nimodipine is in the 0.1 to 1 nM range, provided that the protein concentration used in the binding assay is not too high. (For early references, see ref. 1072; additional recent studies are listed in table 8). The binding detected in skeletal muscle is consistently of somewhat lower affinity (K_d 1 to 5 nM). (+)-[^{3}H]PN 200-110 is an optically pure radioligand which has a very high affinity for the dihydropyridine receptor (K_d 0.01 to 0.1 nM in brain and heart tissue) and offers the advantage of particularly low nonspecific binding. With [^{3}H]Bay K 8644, a K_d of 30 nM was initially reported for cultured cardiac myocytes (61), but lower values (2 to 3 nM) have been obtained with membrane preparations (see table 8).

The number of [^{3}H]nitrendipine specific binding sites (B_{max}) has been compared in total membrane preparations from various tissues (458). It is much higher in

TABLE 8

*Dissociation constants (K_d) of [^{3}H]dihydropyridines in membranes from various tissues**

Tissue	K_d (nM)	Temperature (°C)	Ref.
[^{3}H]Nitrendipine			
Mouse brain	0.33	25	(891)
Bovine aorta	0.16	25	(953)
Rabbit aorta	1.6	30	(108)
Bovine coronary artery	0.22	23	(794)
Rat myometrium	0.14	25	(468)
Rabbit myometrium	0.46	25	(767)
Bovine trachea	0.15	25	(151)
Rat seminal vesicles	0.41	23	(881)
Rabbit heart	0.12	25	(699)
Chick heart	0.4	4	(900)
Rat adrenal cortex	0.26	25	(284)
Rat adenohypophysis	0.7–1.1	25	(778)
Bovine adenohypophysis	0.14	37	(1061)
[^{3}H]Nimodipine			
Rabbit skeletal muscle	2.4	10	(637)
	3.2	37	
Human heart	0.23	37	(277)
(+)-[^{3}H]PN 200-110			
Rabbit skeletal muscle	0.2	4	(92)
Rat brain	0.035	37	(672)
	0.08	23	(1035)
	0.016	20	(1138)
Rat heart	0.064	37	(672)
Chick heart	0.06	20	(902)
[^{3}H]Bay K 8644			
Rabbit brain	3	15	(565)
Rabbit heart	2.4	15	(565)
Frog skeletal muscle	1.8	20	(549)

* Only recent data are listed in this table. For earlier references, see Triggle and Janis (1072).

TABLE 9

Number (B_{max}) of [^{3}H]nitrendipine binding sites in various membrane preparations

Membrane preparation	B_{max} (pmol/mg protein)	Ref.
"Total" membranes from guinea pig		(458)
Heart	0.21	
Cerebral cortex	0.17	
Ileum	0.094	
Skeletal muscle	1.1	
"Ryanodine-sensitive" sarcoplasmic reticulum subfraction from dog heart	1.5	(1162)
Sarcolemma		
Dog heart	1.0–1.4	(952, 1089)
Rabbit heart	2.8	(103)
Plasma membrane fraction		
Rat myometrium	0.72	(468)
Rat gastric fundus	0.43	(468)
Guinea pig ileal smooth muscle	2.3	(440)
Rat aorta	0.5	(1157)
T-tubules from rabbit skeletal muscle	50	(341)

skeletal muscle than in heart, cerebral cortex, or ileum (table 9). When purified membrane fractions are considered, T-tubules from skeletal muscle present a density of binding sites of 50 pmol/mg of protein, i.e., 20 to 50 times the highest values found in isolated membranes from other tissues (table 9). In a comparative study on skeletal muscle membranes, Ferry et al. (279) found that the B_{max} depended on the particular tritiated ligand used. However, in other studies, similar B_{max} values were obtained with different ligands: [^{3}H]PN 200-110 and [^{3}H] nitrendipine (672); [^{3}H]dihydropyridine isothiocyanate and [^{3}H]nitrendipine (462, 1118); [^{3}H]Bay K 8644 and [^{3}H]nitrendipine (565).

Competition studies with unlabelled dihydropyridine derivatives have generally supported the concept of a single class of high-affinity binding sites (Hill coefficient close to 1). The use of enantiomeric pairs of dihydropyridines has confirmed the specificity of binding by demonstrating its stereoselectivity (table 10). The affinity ratio of isomers is only 3 to 5 for nimodipine, but it may reach 600 for Bay e 6927.

Kinetic measurements with [^{3}H]nitrendipine have yielded values of 2 to 6 × 10^{-8} $M^{-1}min^{-1}$ for the association rate constant (k_1, 25°C) in brain (1177), ileum (82), aorta, and heart (953). The dissociation rate constant (k_{-1}) ranges from 0.02 to 0.06 min^{-1}. K_d values derived from rate measurements and from equilibrium studies are in good agreement. In a recent detailed kinetic study using [^{3}H]nitrendipine and (+)-[^{3}H]PN 200-110, Weiland and Oswald (1138) observed biphasic pseudo-first order association time courses with both radioligands and monophasic dissociation kinetics with (+)-[^{3}H]PN 200-110. They found a biphasic dissociation with [^{3}H] nitrendipine, which they attributed to the racemic nature of this radioligand. According to the model of these authors, the dihydropyridine binding site in brain membranes exists in two interconvertible states before ligand binding: one that binds the radioligand with high affinity and one that has no detectable affinity for dihydropyridines.

The effect of temperature on equilibrium binding parameters for [^{3}H]nitrendipine binding to rat brain synaptosomes has been investigated by Boles et al. (80). K_d (nM) decreased from 0.47 at 37°C to 0.17 at 25°C and 0.12 at 0°C. B_{max} did not vary significantly with temperature. The dissociation rate constant (k_{-1}) of [^{3}H]nimodipine binding to guinea pig brain membranes was 6-fold higher at 37°C than at 25°C (386).

Occasionally, a second [^{3}H]nitrendipine binding site, of lower affinity, has been found in heart (63, 1089, 1090), arteries (1070), and cultured cardiac myocytes (720). A low-affinity binding site, which is difficult to detect for technical reasons, might be functionally relevant in heart (see below). Two binding sites for Bay K 8644 in heart have also been postulated by Vaghy et al. (1089, 1090).

Recently, a [^{3}H]nimodipine binding site has been char-

TABLE 10
Stereoselectivity of dihydropyridine binding

Tissue	Labelled ligand*	IC_{50}† or K_i‡(nM) (−)	(+)	Ref.
Nitrendipine				
Rat brain	NIM	0.34	8.8	(64)
Guinea pig brain	NIM	0.24	32	(389)
Nimodipine				
Rat brain	NIM	1.0	2.4	(64)
Guinea pig ileum	NIT	0.47	2.3	(440)
Nicardipine				
Guinea pig brain	NIM	28	2.3	(273)
Rat brain	PN	2.9	0.38	(672)
Rat heart	PN	4.3	0.46	(672)
Guinea pig skeletal muscle	IO	42	7	(276)
PN 200-110				
Guinea pig skeletal muscle	NIM	206	2.2	(274)
	PN	375	1.3	(448)
	IO	198	2.0	(276)
Rat brain	PN	15	0.06	(672)
Rat heart	PN	20	0.12	(672)
Human heart	NIM	24	0.2	(277)
Bay e 6927				
Bovine aorta	NIT	0.025	15	(953)
(−)(*R*)202-791 and (+)(*S*)202-791				
Guinea pig heart	NIT	0.9	90	(1161)
Dog heart	NIT	1.6	230	(1161)

* Abbreviations: NIM, [^{3}H]nimodipine; NIT, [^{3}H]nitrendipine; PN, (+)-[^{3}H]PN 200-110; IO, [^{125}I]iodipine.

† IC_{50}, concentration of unlabelled dihydropyridine (−)- or (+)-isomer inhibiting by 50% the binding of labelled ligand.

‡ K_i, inhibition constant of unlabelled dihydropyridine isomer.

acterized in human red blood cells (1029, 1031). This site presented unusual features, in particular a relatively low affinity (K_d 52 nM) and an inverse stereoselectivity with respect to the "classical" dihydropyridine binding site, which is lacking in red blood cells. The significance of this site has been discussed in section II F.

The role of divalent cations in dihydropyridine binding was revealed by experiments using chelating agents. When membrane fractions are prepared in the absence of chelating agents, addition of Ca^{2+} at "physiological" concentrations does not influence binding. In contrast, addition of EDTA or EGTA to the incubation medium or pretreatment of membranes with these agents inhibits binding, usually by reducing B_{max} without affecting K_d (457, 458, 699), or by converting high-affinity sites into low-affinity sites (chick heart) (884). The only exception appears to be the binding in skeletal muscle T-tubules, which is unaffected by EGTA (259, 341), or even stimulated 30% by EDTA or EGTA (458). However, after solubilization of skeletal muscle binding sites with digitonin, the binding was abolished by 0.1 mM EDTA and markedly reduced by EGTA (385).

The inhibitory action of chelators is prevented or reversed by divalent cations, in particular Ca^{2+} (61, 62, 268, 388, 457, 458, 699). With EDTA-treated membranes from heart and smooth muscle, the apparent K_{Ca} was 3 to 5 μM in the study of Luchowski et al. (699). At a concentration of 1 mM, Sr^{2+} or Mn^{2+} was as effective as Ca^{2+}, whereas Mg^{2+}, Ba^{2+}, Co^{2+}, or Ni^{2+} only partially restored binding. In brain membranes, La^{3+} (0.1 mM), Cu^{2+} (1 mM), Co^{2+} (1 mM), and Mn^{2+} (10 mM) inhibited Ca^{2+}-dependent stimulation of binding (457). Gould et al. (457) have pointed out that the ability of various cations to stimulate [^{3}H]nitrendipine binding or to block the stimulatory action of Ca^{2+} correlates with their agonist or antagonist profile on calcium channels. Sr^{2+} and Ba^{2+} support [^{3}H]nitrendipine binding and permeate the channel, whereas La^{3+}, Cu^{2+}, Co^{2+}, and Mn^{2+} antagonize Ca^{2+}-dependent binding and the Ca^{2+} current.

b. PHENYLALKYLAMINE BINDING. Among tritiated phenylalkylamines, (−)-[^{3}H]desmethoxyverapamil appears to be a particularly useful ligand, exhibiting higher affinity and lower nonspecific binding than (−)-[^{3}H]verapamil (278). The binding of phenylalkylamines has been most thoroughly studied in skeletal muscle (360–362, 447). In this tissue, as well as in mammalian heart, the K_d of [^{3}H]verapamil is 20 to 50 nM. The K_d of (−)-[^{3}H]desmethoxyverapamil is lower: 1 to 2 nM in guinea pig hippocampus membranes (278) and rabbit skeletal muscle T-tubules (362). The B_{max} is markedly temperature dependent (447, 910). With skeletal muscle membranes, the B_{max} of [^{3}H]verapamil at 37°C was only 20 to 25% of that measured at 2–10°C. The B_{max} at low temperature has been reported to be equal to that of dihydropyridine ligands (360, 361), or twice as high (278, 447). According to Garcia et al. (368), the stoichiometric ratio (verapamil sites/nitrendipine sites) would tend to 1 in purified cardiac sarcolemma, as compared to 3 to 4 in crude membrane fractions. These authors suggest that a sizeable part of the verapamil binding sites in heart is located in a nonsarcolemmal, nonmitochondrial membrane fraction, devoid of dihydropyridine binding sites.

The binding of verapamil or desmethoxyverapamil is displaced by D 600, the (−)isomer being 3- to 10-fold more potent than the (+)isomer (278, 360, 447). The selectivity is less obvious with verapamil stereoisomers. The rate of dissociation of the preformed [^{3}H]verapamil-T-tubule complex is increased by high concentrations (0.5 to 10 μM) of unlabelled verapamil (361, 447). Possibly, saturation of a second, low-affinity binding site for verapamil accelerates dissociation of [^{3}H]verapamil from its high-affinity binding site.

In contrast to dihydropyridine binding, the binding of verapamil is depressed by divalent cations. According to Reynolds et al. (910), detection of [^{3}H]verapamil binding in brain membranes requires pretreatment with EDTA, and the binding is maximal at 1 to 10 μM Ca^{2+}. More extensive data have been obtained on skeletal muscle, using [^{3}H]verapamil (361, 447) and (−)-[^{3}H]desmethoxyverapamil (362). In this latter study, the IC_{50} of Ca^{2+}, the most potent inhibitor, was 0.5 μM, and Sr^{2+}, Ba^{2+}, and

Mg^{2+} were less potent than Ca^{2+}. The apparent affinity for Ca^{2+} of the inhibitory site is close to that of the high-affinity Ca^{2+} coordination site found in electrophysiological experiments. It is thus likely that this coordination site influences the binding of the phenylalkylamines (362).

In addition, monovalent cations including K^+ and Na^+ inhibit verapamil binding to skeletal muscle T-tubules at concentrations greater than 10 mM, whereas 10-fold higher concentrations do not affect [^{3}H]nitrendipine binding (341). Clearly, physiological cation concentrations are not optimal for verapamil binding.

GTP and ATP partially inhibit [^{3}H]verapamil binding to T-tubules, half-maximal inhibition being observed at 0.1 to 0.3 mM (361). Nucleotides decrease B_{max} with no change of K_d and increase the proportion of verapamil-receptor complex dissociating at a fast rate. Transformation of high-affinity sites into low-affinity sites probably accounts for the B_{max} effect.

c. DILTIAZEM BINDING. Glossmann et al. (392) succeeded in measuring specific, reversible binding of *d-cis*-[^{3}H]diltiazem to skeletal muscle membranes. The binding was stereoselective, *l-cis*-diltiazem being much less potent as a displacing agent than *d-cis*-diltiazem. Glossmann et al. (392) reported a K_d of about 40 nM and a B_{max} that decreased markedly at temperatures above 10°C. At low temperature the number of sites amounted to half that of [^{3}H]nitrendipine binding sites. However, in their T-tubule preparation, Galizzi et al. (362) found a B_{max} (at 25°C) of 50 pmol/mg of protein, i.e., similar to the numbers of binding sites for [^{3}H]nitrendipine, (−)-[^{3}H]desmethoxyverapamil, and [^{3}H]bepridil. In brain and cardiac membranes, the number of diltiazem binding sites (at 25–30°C) was also found to be about the same as that of dihydropyridine binding sites (40, 965).

An inhibitory effect of Ca^{2+} on [^{3}H]diltiazem binding has been demonstrated in cardiac membranes (40) and in skeletal muscle membranes (362). In this latter study, [^{3}H]diltiazem, (+)-[^{3}H]desmethoxyverapamil, and [^{3}H]bepridil binding sites were modulated by divalent cations in a very similar fashion.

d. FLUNARIZINE BINDING. Preliminary data (433, 434) obtained on rat aorta (whole tissue and microsomal fraction) suggest the existence in this tissue of a saturable and reversible binding of [^{3}H]flunarizine, the concentration for half-saturation being about 100 nM (434). Further work is necessary to better characterize this binding.

2. Pharmacological specificity and interactions between different chemical classes of calcium entry blockers. a. DIHYDROPYRIDINE BINDING. **i. Pharmacological specificity.** Several studies have examined the action on dihydropyridine binding of drugs that do not have the calcium channel as primary target (63, 82, 341, 457, 717). The binding is little sensitive to a variety of receptor-active ligands (adenosine, adrenergic, cholinergic, histamine, phencyclidine, opiate, GABA, and benzodiazepine receptors) and is unaffected by agents acting on Na^+ channels (tetrodotoxin, batrachotoxin) and K^+ channels (4-aminopyridine, apamin), antiarrhythmics (quinidine, lidocaine, procainamide), and intracellular Ca^{2+} antagonists (TMB-8). However, the binding is inhibited by veratridine (IC_{50} 5 μM) (82) and by amiodarone (K_i 1 μM) (341).

Gould et al. (456) found that antischizophrenic drugs of the diphenylbutylpiperidine class (pimozide, fluspirilene, penfluridol, and clopimozide) inhibit [^{3}H]nitrendipine binding to brain membranes with IC_{50} values of 10 to 30 nM. These authors suggested that a calcium channel blocking property might be involved in the ability of this class of neuroleptics to relieve negative symptoms of schizophrenia (emotional withdrawal).

Drugs such as pimozide and penfluridol are also rather potent calmodulin antagonists. Luchowski et al. (699) reported a good correlation between the abilities of a series of drugs to antagonize calmodulin-dependent phosphodiesterase and to inhibit [^{3}H]nitrendipine binding to cardiac and smooth muscle membranes. This correlation does not necessarily indicate that calmodulin is involved in the binding of dihydropyridines, since hydrophobic interactions of limited specificity are known to be of importance in the action of calmodulin antagonists (see ref. 930 and section III C).

ii. Effect of phenylalkylamines. As illustrated in fig. 8a, phenylalkylamines inhibit dihydropyridine binding in a concentration-dependent fashion, but the inhibition reaches a maximum of 30 to 60% in most studies on brain, heart, and smooth muscle membranes (table 11). (−)D 600 is distinctly more potent than (+)D 600. While being less potent than (−)verapamil, (+)verapamil is able to inhibit a much higher proportion of [^{3}H] nimodipine binding to human heart membranes (277). In skeletal muscle, it seems that (+)isomers are more effective displacers of dihydropyridine binding than (−)isomers, at least in the absence of diltiazem (92, 274).

The level of maximal inhibition by phenylalkylamines is higher with an analogue such as tiapamil (795), and it depends on the concentration of [^{3}H]dihydropyridine used (80, 242, 458). If this concentration is low with respect to the K_d of [^{3}H]nitrendipine, maximal inhibition by verapamil or D 600 may approach 100%. Scatchard analysis of [^{3}H]dihydropyridine binding in the presence of increasing concentrations of phenylalkylamines has shown that these drugs increase the apparent K_d, but that this augmentation does not exceed a factor of 2 to 5, in contrast to what is observed in true competitive inhibition (80, 82, 242, 458). In kinetic experiments, the dissociation rate constant of the [^{3}H]dihydropyridine-receptor complex is increased (80, 242, 795). Thus, it is clear that verapamil and D 600 influence dihydropyridine binding by acting at a distinct site and should be classified as negative heterotropic (allosteric) effectors.

iii. Effect of diltiazem. Conflicting results (inhibi-

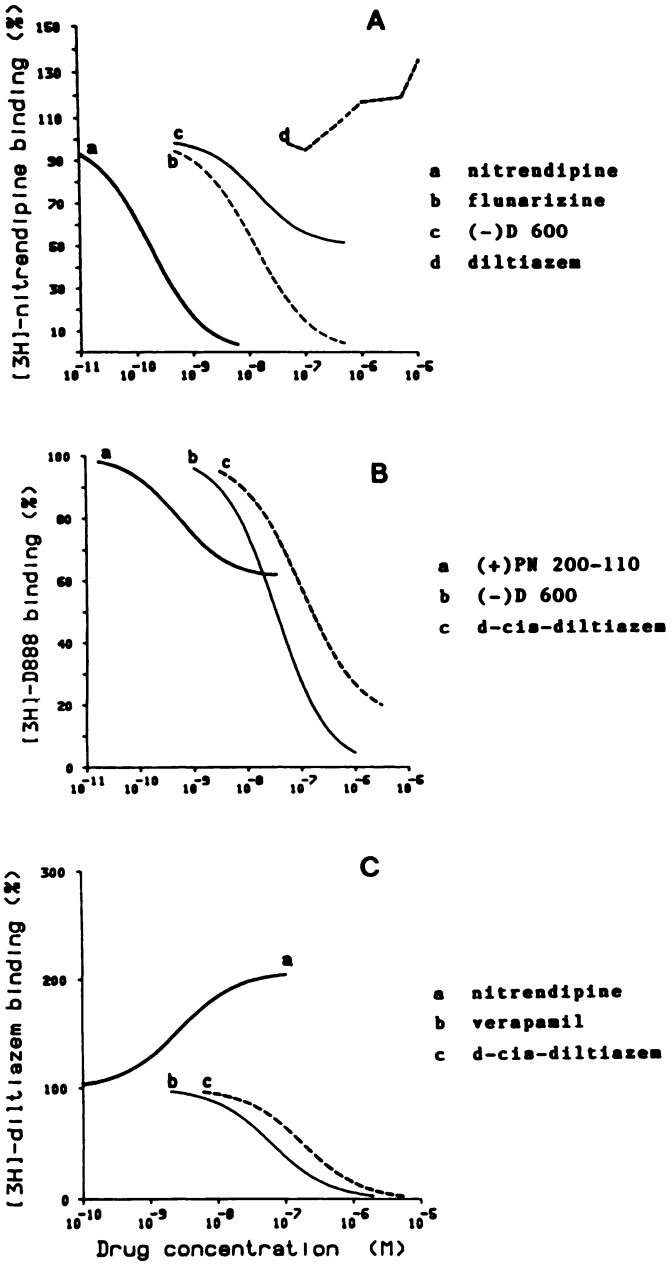

FIG. 8. *A*, effect of various calcium entry blockers on specific binding of [^{3}H]nitrendipine (0.055 nM) in a microsomal fraction from guinea pig ileal smooth muscle. *Curves a, b,* and *c* were drawn from the IC_{50} values and Hill coefficients reported by Bolger et al. (82), assuming that the maximal inhibition of binding was 100% for nitrendipine and flunarizine and 50% for (−)D 600. *Curve d* shows experimental data from the same authors. *B*, effect of various calcium entry blockers on specific binding of (−)-[^{3}H]desmethoxyverapamil (*D888*) (2 to 3 nM) in isolated membranes from guinea pig hippocampus. Curves were drawn from the K_i or IC_{50} values and Hill coefficients reported by Ferry et al. (278), assuming a maximal inhibition of 39%, 100%, and 85% for (+)PN 200-110, (−)D 600, and *d-cis*-diltiazem, respectively. *C*, effect of various calcium entry blockers on specific binding of *d-cis*-[^{3}H] diltiazem (4 nM) in isolated membranes from rat cerebral cortex. Curves were drawn from values of concentrations eliciting half-maximal stimulation or inhibition of binding at 37°C, assuming a maximal stimulation of 108% for nitrendipine and a maximal inhibition of 100% for verapamil and *d-cis*-diltiazem, as reported by Schoemaker and Langer (965).

TABLE 11
Inhibition of [^{3}H]nitrendipine or [^{3}H]nimodipine binding by verapamil and D 600

Tissue	Drug*	Maximal inhibition (%)	IC_{50} (nM)†	Ref.
Brain				
Rat	(±)V	60	280	(717)
	(±)V	64	11	(242)
	(±)D	30	42	(242)
	(±)V	65	180	(1177)
	(±)V	100	400	(64)
Guinea pig	(−)D	50–60	26	(389)
	(±)D	25	10	(795)
	(±)V	40–50	28	(458)
Heart				
Rat	(±)V	75	38	(242)
	(±)D	50	28	(242)
Guinea pig	(±)V	40–50	28	(458)
	(±)D	40–50	12	(458)
Rabbit	(−)D	50	6	(564)
	(+)D	50	450	(564)
Human	(−)V	18	123	(277)
	(+)V	87	630	(277)
Ileum				
Guinea pig	(+)V	63	10	(82)
	(±)D	51	27	(82)
	(−)D		14	(82)
	(+)D		200	(82)
	(±)V	40–50	21	(458)
	(±)D	40–50	4	(458)

* Abbreviations: V, verapamil; D, gallopamil (D 600).
† Concentration giving half-maximal inhibition.

tion, no effect, or stimulation) were initially reported for the effect of diltiazem on [^{3}H]dihydropyridine binding. The situation was clarified by the finding of a marked influence of temperature on the action of *d-cis*-diltiazem, the pharmacologically active isomer (80, 207, 209, 276, 392, 533). At 0°C, inhibition is usually observed. Stimulation of binding by *d-cis*-diltiazem (fig. 8a), but not by *l-cis*-diltiazem, occurs at concentrations of 0.1 to 100 μM, may reach 100 to 120%, and is more important at 37°C than at 25°C. This action may be considered as a positive allosteric regulation of dihydropyridine binding.

With brain membranes, the stimulatory effect of diltiazem has been consistently ascribed to a reduction of the K_d of dihydropyridine binding, with no modification of B_{max} (table 12). The reverse situation prevails in studies dealing with skeletal muscle T-tubules and intestinal smooth muscle (increase in B_{max} and no effect on K_d). Results obtained on cardiac muscle are contradictory. When dissociation experiments have been carried out, *d-cis*-diltiazem has been found to decrease k_{-1}, irrespective of the tissue (80, 273, 274, 448, 566, 746, 1177). Thus, when diltiazem appears to be acting on B_{max}, it could actually be converting undetected low-affinity dihydropyridine binding sites into high-affinity sites. A

similar mechanism of action has been postulated for divalent cations (see above).

A number of other drugs have been reported to stimulate dihydropyridine binding (see table 13). They include two Ca^{2+} entry blockers, fostedil and bepridil. MDL 12330A is a novel lactamimide compound that possesses negative inotropic and chronotropic properties and is more potent than *d-cis*-diltiazem at enhancing [^{3}H]nitrendipine binding (671).

iv. Effect of diphenylpiperazines and other drugs. Flunarizine (fig. 8a), cinnarizine, lidoflazine, prenylamine, and Hoe 263 (689) are able to completely displace dihydropyridines from their binding site with Hill coefficients of 0.7 to 1 (64, 82, 534, 795). Various authors have reported widely different apparent K_i values for these drugs. As illustrated in fig. 9, the apparent potency of flunarizine seems to correlate with the protein concentration used in the binding assay. Probably, at high tissue concentration, the free concentration of this highly lipophilic compound is distinctly lower than its nominal concentration (see also refs. 1072 and 1138).

At high [^{3}H]dihydropyridine concentrations, maximal displacement by diphenylpiperazines may be less than 100% (566, 795). Like phenylalkylamines, these drugs accelerate the dissociation of dihydropyridines from their receptor (746, 795) and could, thus, also act allosterically.

Gould et al. (458) reported that [^{3}H]nitrendipine binding in skeletal muscle membranes is more sensitive to inhibition by diphenylpiperazines than it is in other tissues (heart, cerebral cortex, and ileum), whereas Eh-

TABLE 12

Effect of d-cis-diltiazem on [^{3}H]dihydropyridine binding

Ligand*	Temperature (°C)	K_d (nM)		B_{max} (pmol/mg)		Ref.
		Control	Diltiazem	Control	Diltiazem	
Brain						
Guinea pig, membranes						
NIM	37	0.62	0.21	0.57	0.45	(273)
Rat, synaptosomes						
NIT	0	0.12	0.23	0.11	0.12	(80)
	25	0.17	0.12	0.11	0.11	
	37	0.47	0.26	0.12	0.12	
Rat, membranes						
PN	37	0.035	0.02	0.22	0.23	(672)
Skeletal muscle						
Guinea pig, membranes						
NIF	37	4.9	5.8	3.9	8.6	(279)
NIT		3.6	2.6	7.0	14.7	
NIM		3.6	2.2	8.0	14.3	
PN		1.4	1.5	20.6	25.4	
Rabbit, T-tubules†						
NIM	10	2.4	2.7			(637)
	37	3.2	2.9			
NIT	10	1.7	1.4			
	37	4.3	2.4			
Rabbit, membranes						
NIT	25	0.75	0.82	3.2	3.9	(566)
	37	0.94	1.7	0.53	2.7	
Cardiac muscle						
Dog, sarcolemma						
NIM	37	0.23	0.2	0.4	0.9	(207)
Guinea pig, membranes						
NIM	37	0.26	0.26	0.33	0.42	(275)
Rabbit, microsomes						
NIT	25	0.19	0.16	0.25	0.26	(566)
	37	0.29	0.18	0.12	0.14	
Rat, membranes						
PN	37	0.064	0.043	0.15	0.18	(672)
NIT	22	1.2	0.7	0.17	0.17	(1058)
Human, membranes						
NIM	37	0.32	0.4	0.107	0.132	(277)
Smooth muscle						
Guinea pig ileum, microsomes						
NIT	25	0.16	0.17	1.1	1.6	(82)

* Abbreviations: NIM, [^{3}H]nimodipine; NIT, [^{3}H]nitrendipine; PN, (+)-[^{3}H]PN 200-110; NIF, [^{3}H]nifedipine.

† B_{max} values were increased 1.5- to 2-fold by diltiazem.

TABLE 13
Drugs resembling d-cis-diltiazem in their action on dihydropyridine binding

Drug	Tissue	Ref.
Calcium antagonists		
Bepridil	Brain	(795)
	Ileal smooth muscle	(82)
Fostedil (KB 944)	Heart	(208, 533)
Neuroleptics		
Mesoridazine, thioridazine, chlorpromazine	Brain	(795)
	Heart	(1058)
Antihistaminics (H_1)		
Dimethindene, chlorpheniramine, pyribenzamine, triprolidine	Brain	(795)
	Ileal smooth muscle	(82)
Muscarinic antagonists		
Biperiden	Brain	(795)
Alpha-adrenoceptor antagonists		
Phentolamine, yohimbine	Ileal smooth muscle	(82)
Miscellaneous		
Phencyclidine	Brain	(83)

lert and Yamamura (243) found that these drugs are more potent in the ileum than in heart or brain.

v. Interactions between phenylalkylamines, diphenylpiperazines, and diltiazem. Murphy et al. (795) have shown that inhibition of [^{3}H]nitrendipine binding by diphenylpiperazines or tiapamil can be antagonized by D 600, an inhibitor of low "efficacy" (which reduced [^{3}H]nitrendipine binding to brain membranes by about 25% at the most), or by diltiazem. Moreover, the inhibitory action of verapamil can be abolished by high concentrations of diltiazem, and the stimulatory action of diltiazem is counteracted by high concentrations of verapamil (80). The inhibition of [^{3}H]nitrendipine binding by diphenylbutylpiperidine neuroleptics (456) or suloctidil (148) is also antagonized by diltiazem or D 600.

b. PHENYLALKYLAMINE BINDING. **i. Pharmacological specificity.** Verapamil binding also displays little sensitivity to drugs acting on other receptors or channels (278, 361, 368, 447, 910). Calmodulin antagonists (trifluoperazine, pimozide, and W-7) are inhibitory (IC_{50} 150 to 300 nM) (447). Among alpha-adrenoceptor antagonists, phentolamine and WB 4101 inhibit binding in the μM range; hydrophobic beta-adrenoceptor antagonists also exhibit weak, nonstereoselective inhibition.

ii. Effect of dihydropyridines. These drugs inhibit [^{3}H]verapamil binding stereoselectively and at concentrations that correspond to their affinities for the dihydropyridine binding site in skeletal muscle (360, 447), cardiac muscle (368), or hippocampal membranes (278). The maximal inhibition is less than 100% (see fig. 8b). Galizzi et al. (360, 361) found that nitrendipine behaves as an apparently noncompetitive inhibitor of [^{3}H]verapamil binding and does not affect the dissociation rate

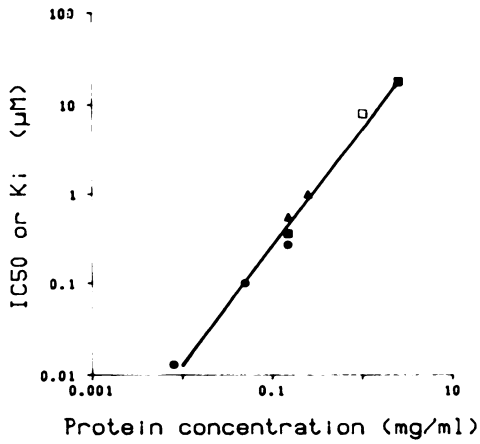

FIG. 9. Effect of protein concentration in assay medium on the apparent potency of flunarizine as inhibitor of [^{3}H]nitrendipine (*closed symbols*) or [^{3}H]verapamil (*open symbol*) binding in membrane preparations. Data were taken from studies on ileal smooth muscle (●) (82, 440, 458), heart (□, ■) (368, 458, 534), and brain (▲) (64, 458).

of bound verapamil. In contrast, Glossmann and coworkers (278, 447) reported that (+)PN 200-110 changed both K_d and B_{max} and increased k_{-1}, which corresponds to a "partial, mixed" type of inhibition. Garcia et al. (368) observed that the level of maximal inhibition by nitrendipine of [^{3}H]verapamil binding to cardiac membranes is related to the composition of the subcellular fraction used. They found no inhibition by nitrendipine (or diltiazem) of [^{3}H]verapamil binding to a "high-density" membrane fraction. They suggested that part of the verapamil receptors in heart is located in membranes of intracellular origin, where they are not coupled with dihydropyridine receptors.

iii. Effect of diltiazem. According to Galizzi et al. (360), *d-cis*-diltiazem behaves like a competitive inhibitor of [^{3}H]verapamil binding to T-tubules (K_i 200 nM). Bepridil behaved similarly, with a K_i of 40 nM. However, using (−)-[^{3}H]desmethoxyverapamil and hippocampal membranes (fig. 8b), Ferry et al. (278) showed that increasing concentrations of *d-cis*-diltiazem caused a limited increase in apparent K_d and at higher concentrations a decrease in B_{max} (mixed type of inhibition). At a rather high concentration (10 μM), diltiazem, like verapamil (see above), accelerated the dissociation of bound [^{3}H]verapamil (361, 447).

iv. Effect of diphenylpiperazines. Flunarizine, cinnarizine, and lidoflazine completely inhibit [^{3}H]verapamil binding to cardiac membranes with apparent K_i values of 4 to 8 μM (368).

c. DILTIAZEM BINDING. As already mentioned, the effect of diltiazem on [^{3}H]dihydropyridine binding is temperature dependent. Likewise, dihydropyridines inhibit *d-cis*-[^{3}H]diltiazem binding to skeletal muscle membranes at low temperatures, but stimulate binding at higher temperatures by increasing B_{max} (392). The stimulation (at 30°C) may reach 100 to 150% and is stereoselective. Interestingly, Bay K 8644 is only a weak stimulator. The inhibitory effect (at 2°C) is not complete (60

to 80%). In contrast to dihydropyridines, phenylalkylamines inhibit binding at all temperatures. Similar results were obtained with brain membranes (fig. 8c) (965). However, in this tissue, dihydropyridines enhance [^{3}H] diltiazem binding (at 30–37°C) by decreasing the dissociation rate of the radioligand.

3. Subcellular localization. a. DIHYDROPYRIDINE BINDING. Subcellular localization studies indicate that specific dihydropyridine binding is essentially associated with the plasma membrane in smooth muscle (fig. 10) (440, 468). In skeletal muscle, the binding is found predominantly in the T-tubule fraction, which is also enriched in ouabain binding sites (341, 387). The presence of [^{3}H] nitrendipine binding sites in the "heavy" sarcoplasmic reticulum fraction of skeletal muscle (259) might reflect its contamination by T-tubule fragments. In cardiac tissue, binding sites are abundant in sarcolemmal fractions (103, 952), but also in particular sarcoplasmic reticulum fractions that appear to be devoid of other plasmalemmal receptors or enzyme markers (208, 1162). According to Brandt (103), these "microsomal" sites could be located in T-tubule membranes physically linked to the junctional sarcoplasmic reticulum. Autoradiographic studies on [^{3}H]nitrendipine binding in rat brain indicate a preferential localization to synaptic zones (796).

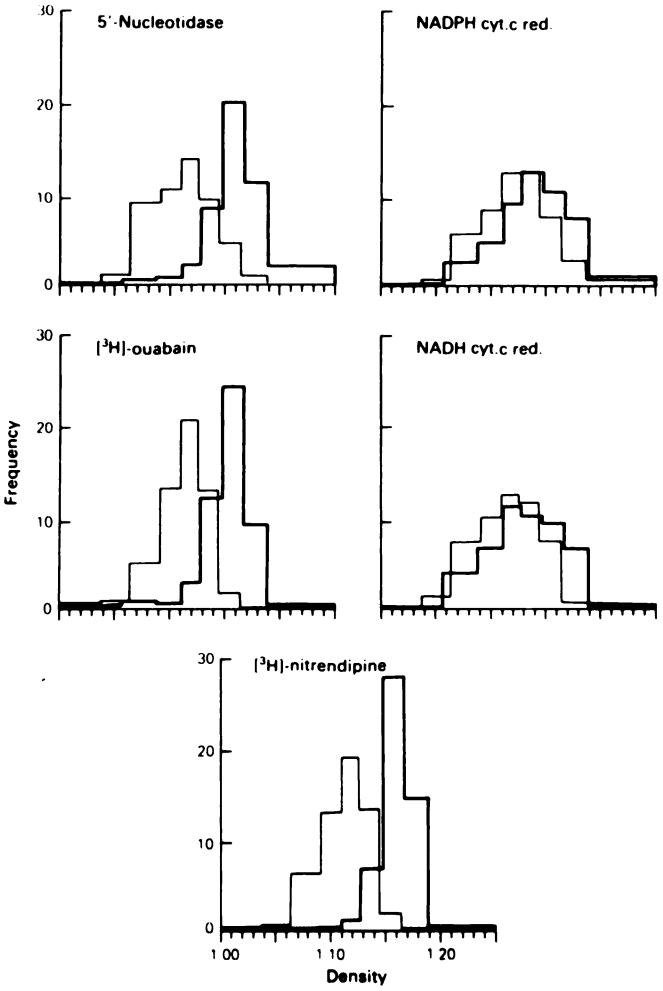

FIG. 10. Density distribution histograms obtained from a microsomal fraction isolated from guinea pig intestinal smooth muscle. *Thin-line histograms* are from an untreated microsomal fraction, and *thick-line histograms* from a fraction pretreated with digitonin. Plasma membranes (5'-nucleotidase, [^{3}H]ouabain) were shifted towards high-density subfractions after digitonin treatment, whereas endoplasmic reticulum membranes (NADPH cytochrome *c* reductase, NADH cytochrome *c* reductase) were hardly shifted. The behavior of [^{3}H]nitrendipine binding sites was very similar to those of 5'-nucleotidase and [^{3}H]ouabain binding sites, indicating that [^{3}H]nitrendipine binding sites are localized in plasma membranes. For further details, see ref. 440. Reproduced from Godfraind and Wibo (440).

b. VERAPAMIL AND DILTIAZEM BINDING. In skeletal muscle, [^{3}H]verapamil and [^{3}H]diltiazem binding sites are also concentrated in T-tubule fractions (360, 392, 447). Heart sarcolemma contains verapamil binding sites, but an important proportion of verapamil receptors have been reported to be associated with a high-density, nonmitochondrial, nonsarcolemmal membrane fraction devoid of dihydropyridine or diltiazem receptors (368). These data suggest that verapamil and diltiazem bind to distinct molecular entities, which are also distinct from the dihydropyridine binding site.

4. Chemical and physical characterization of binding sites. a. DIHYDROPYRIDINE BINDING. **i. Chemical properties.** Dihydropyridine binding is heat sensitive: it is, for example, lost after 30 min at 55°C (717). It is abolished by treatment with various proteases, phospholipase A_2, or phospholipase C (82, 341, 388, 717). Thus, the receptor protein might be stabilized by phospholipid. The solubilized and purified binding site is retained by lectin columns, which suggests that it might be a glycoprotein (93, 183, 717). The inhibitory action of dithiothreitol indicates that a sulfhydryl group influences the affinity of the binding site (104, 717). The effect of pH on [^{3}H] nitrendipine binding to T-tubules suggests the existence of an essential ionizable group with a pK_a of 5.4 (341).

ii. Molecular weight and subunit structure. The technique of target size analysis by radiation inactivation on intact membranes has been used to estimate the size of the entity that binds dihydropyridines. Results obtained by 3 different groups indicate a M_r of about 200,000 for the binding site ([^{3}H]nitrendipine or [^{3}H] nimodipine) in brain or skeletal muscle membranes (280, 448, 831) and of 280,000 in ileal smooth muscle (1118). Oddly enough the apparent molecular size is lower when [^{3}H]PN 200-110 rather than [^{3}H]nimodipine is used as the channel ligand (448). Moreover, preincubation with *d-cis*-diltiazem, but not *l-cis*-diltiazem, reduces the apparent M_r, suggesting that positive allosteric regulation by diltiazem is accompanied by dissociation from the channel of a subunit with a M_r of 60,000 (448).

Another approach has been to label (photoaffinity labeling) irreversibly the binding site in situ and then to estimate by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis the molecular weight of the labelled subunit(s). The main subunit revealed in this

manner has a M_r of 32,000 to 170,000 (124, 281, 359a, 539, 637, 1118).

Several groups have succeeded in solubilizing and purifying the dihydropyridine receptor. After solubilization, stereoselective modulation by calcium channel ligands (including stimulation of binding by diltiazem) was still demonstrable (92, 183, 385). Curtis and Catterall (184) solubilized the [^{3}H]nitrendipine-receptor complex from rabbit T-tubules with digitonin and found that the purified receptor contains 3 polypeptides with M_rs of 160,000 (130,000 after reduction), 50,000, and 33,000. Borsotto et al. (91, 93) also reported substantial purification of the dihydropyridine receptor from the same tissue. The subunit composition they determined seemed to be a polypeptide of M_r 142,000 and a doublet of M_r 32,000 to 33,000. A 1:1:1 complex of these subunits would be consistent with the M_r of 210,000 obtained on the same tissue by radiation inactivation analysis (831). The polypeptide of M_r 142,000 might be a proteolytic product of the entity of M_r 170,000 identified after photoaffinity labeling by Galizzi et al. (359a). On the other hand, in a recent study on chick heart membranes, no polypeptide of $M_r > 60{,}000$ was found (902). Further studies are needed to resolve these discrepancies, which may arise from tissue differences or be artifactual.

iii. Phosphorylation. Nitrendipine, but not Bay K 8644, induces the phosphorylation of a protein of M_r 42,000 in dog cardiac membranes via an endogenous kinase (539). This protein might be identical to the major polypeptide labelled by [^{3}H]dihydropyridine isothiocyanate in these membranes. Phosphorylation of the same protein is enhanced by isoproterenol by a cyclic AMP-independent pathway. This effect of isoproterenol is blocked by propranolol and attenuated by carbachol. Nitrendipine and isoproterenol have opposite pharmacological effects at the cardiac calcium channel, but it is possible that these two compounds stimulate phosphorylation at different sites of the same protein.

Curtis and Catterall (185) reported that the catalytic subunit of cyclic AMP-dependent protein kinase phosphorylates the subunit of M_r 53,000 in intact T-tubule membranes. This protein might be the target for the regulation by cyclic AMP of calcium channel function (906).

b. VERAPAMIL BINDING. Specific binding of verapamil is abolished by trypsin and by heating at 60°C for 15 min (910). Binding is optimal at slightly alkaline pH, the pH dependence suggesting an ionizable group with a pK_a of 6.5 (361). Sulfhydryl blocking reagents depress verapamil binding (447). Target size analysis indicates a M_r of 110,000 for the verapamil receptor (447).

5. Models of calcium channel receptor sites. We would like to summarize and integrate the binding data discussed so far by presenting functional models of calcium channel receptor sites.

When the properties of the dihydropyridine binding site are compared in different tissues, only the skeletal muscle binding site shows distinct features. The density of sites in T-tubules is at least one order of magnitude higher than in all other types of membrane examined so far, and their affinity, at least for antagonist dihydropyridines, is somewhat lower than in other tissues. Interestingly, the ontogenic appearance of these distinctive properties coincides with the establishment of functional nerve muscle contacts (961) (see below). In addition, skeletal muscle dihydropyridine binding sites are less sensitive to treatment with Ca^{2+} chelators. As for the [^{3}H]verapamil and [^{3}H]diltiazem binding sites, too few comparative data are available to draw any conclusion about possible tissue differences.

In view of the obvious structural differences between the various classes of calcium entry blockers, one might expect that their specific binding sites be distinct. It is clear that the dihydropyridine binding site differs from the site(s) that bind(s) [^{3}H]verapamil and [^{3}H]diltiazem. (*a*) The dihydropyridine binding site has a requirement for divalent cations, whereas both other sites are inhibited by Ca^{2+}. (*b*) According to Glossmann and coworkers, decreasing temperature from 37°C to 0°C increases the B_{max} of [^{3}H]verapamil and [^{3}H]diltiazem, while it does not influence the B_{max} of [^{3}H]dihydropyridines, but decreases their K_d. (*c*) The effect of pH indicates that two different ionizable groups are involved in dihydropyridine and verapamil binding. (*d*) Competition experiments clearly demonstrate that the interactions between dihydropyridines, on the one hand, and phenylalkylamines or benzothiazepines, on the other hand, are not purely competitive, but of an allosteric nature.

The relation between [^{3}H]verapamil and [^{3}H]diltiazem binding sites is more controversial. It is not yet clearly established whether the interactions between these two classes of compounds are purely competitive or allosteric. Murphy et al. (795) proposed that all nondihydropyridine calcium entry blockers act at a single site allosterically linked to the dihydropyridine binding site (fig. 11). Alternatively, diltiazem could bind to a distinct site in close contact with the binding site for phenylalkylamines (see fig. 11) (278, 390). Recently, Galizzi et al. (359a) have shown that the binding sites for the various chemical classes of calcium entry blockers are all present in a single polypeptide of M_r 170,000.

The mode of interaction of diphenylpiperazines, such as flunarizine, with the binding sites for the other chemical classes of calcium entry blockers has been investigated in less detail. The effects of flunarizine on [^{3}H] nitrendipine binding resemble those of the phenylalkylamines (see table 14), but under usual experimental conditions, this drug is able to completely inhibit [^{3}H] nitrendipine binding. However, at high radioligand concentration, maximal inhibition by flunarizine or lidoflazine may be less than 100% (566, 795). Flunarizine and related drugs also completely displace [^{3}H]verapamil binding to cardiac membranes (368). Thus, these effects are compatible with the hypothesis that flunarizine and

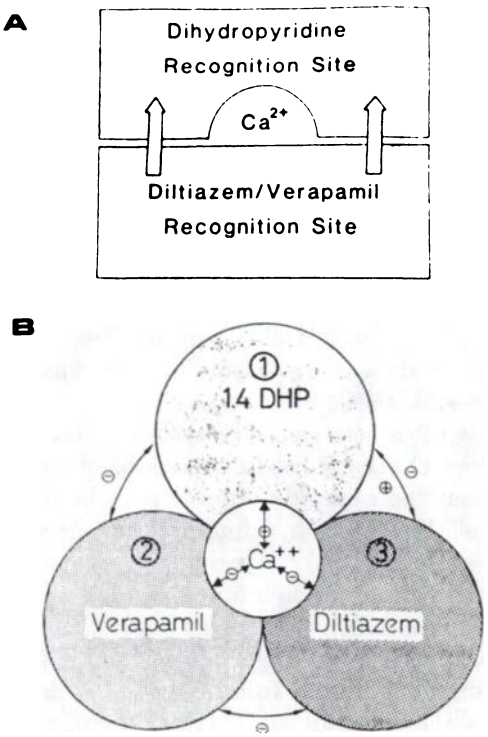

FIG. 11. Models of calcium channel receptor sites for dihydropyridines (*1.4 DHP*), verapamil, and diltiazem. *A*, model of Murphy et al. (795); *B*, model of Glossmann et al. (390). *Arrows* symbolize positive and negative (*plus* and *minus signs*) allosteric interactions between subunits and with Ca^{2+} binding site(s). Reproduced with permission from Murphy et al. (795) and Glossmann et al. (390).

TABLE 14
*Effects of various calcium channel blockers on [^{3}H]nitrendipine binding**

Drug	IC_{50} (nM)	I_{max} (%)	Effect of high radioligand concentration on I_{max}	k_{-1}	Effect of diltiazem on inhibition by drug
Nifedipine	1	100	NE	NE	NE
Verapamil	100	40–80	↘	↗	↘
Flunarizine	100	100	↘	↗	↘
Diltiazem	(Stimulation)			↘	

* Abbreviations: IC_{50}, concentration giving half-maximal inhibition (approximate value); I_{max}, maximal inhibition; k_{-1}, dissociation rate constant; NE, no effect.

related drugs interfere directly with the phenylalkylamine binding site, or the verapamil/diltiazem recognition site in the model of Murphy et al. (795). The fact that diltiazem and D 600 antagonize the inhibitory effect of flunarizine and related drugs on [^{3}H]nitrendipine binding argues against the possibility that these highly lipophilic compounds modify the properties of the binding sites in a purely nonspecific manner.

In experiments on PC_{12} cells, Messing et al. (746) found that phenytoin, at clinically relevant concentrations, inhibited K^+-stimulated ^{45}Ca uptake and decreased the apparent affinity of [^{3}H]nitrendipine for its receptor without affecting its dissociation rate. In addition, diltiazem enhanced the inhibitory effects of phenytoin on [^{3}H]nitrendipine binding, whereas it reversed the inhibitory effects of verapamil and flunarizine and did not affect that of nimodipine. This pattern of effects is thus distinct from those of all known calcium entry blockers.

6. Regulation of binding. The perinatal evolution of [^{3}H]nitrendipine binding sites in several rat tissues has been studied by Kazazoglou et al. (619). B_{max} increased markedly over the perinatal period without modification of K_d. In chick (900, 961) in ovo, the B_{max} and K_d values were similar in embryonic heart and skeletal muscle (K_d 0.4 to 0.5 nM). Skeletal muscle binding sites were synthesized after the ninth day of embryonic development, concomitantly with the differentiation of myotubes, and this phenomenon could be reproduced in cell culture. After hatching, the number of sites increased over a few days, and this increase was much more pronounced in skeletal muscle than in heart. Moreover, in chick skeletal muscle, hatching was accompanied by an immediate rise of K_d to a value of 1.8 nM.

The second phase of appearance of binding sites, which starts near hatching, corresponds to the establishment of functional nerve-muscle contacts. However, denervation of postnatal skeletal muscle increased B_{max} and did not affect K_d (961). Chemical denervation by 6-hydroxydopamine also increased B_{max} in chick heart (900). In contrast, treatment of 7-day-old chicks with reserpine or alprenolol produced a decrease in the number of skeletal muscle nitrendipine receptors and an increase (4- to 5-fold) in their affinity for nitrendipine (962). These effects were prevented by simultaneous injection of isoproterenol. Long-term treatment of myotubes in culture with isoproterenol or other compounds that increased intracellular cyclic AMP led to a large increase in the number of binding sites and to a decrease in their affinity. Besides, treatments that increased cyclic AMP had the immediate effect of enhancing ^{45}Ca entry into myotubes via nitrendipine-sensitive channels. Thus, in chick skeletal muscle, beta-adrenergic stimulation is probably responsible for the neonatal maturation of dihydropyridine receptors. Similar observations have been obtained with some neuronal clonal cell lines (350). In these cells, treatment with agents that increased cyclic AMP induced the appearance of voltage-sensitive calcium channels that were sensitive to nitrendipine, diltiazem, and D 600.

In contrast to the results obtained with chick skeletal muscle, reserpine did not influence the number of [^{3}H]nitrendipine receptors in neonatal chick heart (900) and even increased this number in rat seminal vesicles (a tissue with rich adrenergic innervation) (881). Reserpine might act not only by reducing alpha-adrenergic stimulation, but also by blocking calcium channels (131), thereby leading to their "up-regulation" (881). The observation that, in chick skeletal muscle, reserpine is less effective than alprenolol in reducing the number of dihydropyridine binding sites is in line with this interpretation (962).

[^{3}H]Nitrendipine binding sites were investigated in

heart and brain membranes from normotensive and spontaneously hypertensive rats (SHR) (147, 554). In young SHR, no abnormality was detected in heart, but the number of sites was increased in brain. In old SHR, the number of sites was increased in heart, possibly reflecting the ventricular hypertrophy that occurred in these animals (147). The number of dihydropyridine binding sites is also increased in brain from morphine-tolerant mice (891).

7. Correlation between binding and pharmacological activity. a. SMOOTH MUSCLE. With intestinal smooth muscle, extensive studies have been carried out to compare affinities of dihydropyridine derivatives for their binding site and potencies in pharmacological experiments (81, 82, 440). Essentially 1:1 correlations have been obtained between inhibition of [^{3}H]nitrendipine binding and inhibition of K^+-depolarization-induced mechanical responses. The response evoked by activation of the muscarinic receptor was less sensitive to dihydropyridine inhibitors, so that IC_{50} values for inhibition of the tonic component of contraction were 10-fold greater than the values for inhibition of binding. The *o*-NCS (isothiocyanate) analogue of nifedipine irreversibly blocked both the mechanical response to muscarinic agonists and [^{3}H]nitrendipine binding (1118).

Similar, but less extensive, studies have been performed on arterial (441, 953) and myometrial tissues. With pig coronary arteries, De Pover et al. (209) showed that the K_d of [^{3}H]nitrendipine corresponded to its EC_{50} for relaxation of K^+-depolarized arteries. With rat aorta, the K_i values of several nifedipine analogues, including nimodipine stereoisomers, correlated with their IC_{50} values for inhibition of stimulus-evoked contraction and ^{45}Ca entry (441, 1160). However, with mesenteric arteries from rat or dog, the K_d of [^{3}H]nitrendipine was 20 times lower than the IC_{50} of nitrendipine for inhibition of K^+-evoked contraction (1070). In rat myometrium, the inhibitory potency of dihydropyridines on K^+-stimulated ^{45}Ca uptake and contraction agreed with their binding affinity (50, 469).

The stereoselectivity of D 600 was comparable in [^{3}H] nitrendipine binding experiments (82) on ileal smooth muscle and in mechanical experiments on the same tissue (577). *d-cis*-Diltiazem, the pharmacologically active isomer, enhanced [^{3}H]nitrendipine binding to pig coronary arteries, whereas *l-cis*-diltiazem was inactive (209). The stimulatory effect of *d-cis*-diltiazem on binding occurred in the same concentration range as its relaxant effect on depolarized coronary arteries.

The potency of flunarizine and related drugs as inhibitors of [^{3}H]nitrendipine binding seems to correlate with their pharmacological potency in intestinal smooth muscle (82, 440). However, Spedding and Berg (1013) have shown that Bay K 8644 was unable to antagonize the inhibitory effects of diphenylpiperazines on Ca^{2+}-induced contractions of K^+-depolarized smooth muscle, whereas it prevented or reversed inhibition by dihydropyridines, verapamil, or diltiazem. Moreover, in rat cerebral microvessels, the apparent affinity of flunarizine for the (+)-[^{3}H]PN 200-110 binding site (K_i 200 nM) was much lower than its potency as inhibitor of K^+-depolarization-evoked ^{45}Ca entry (Morel and Godfraind, unpublished data). Thus, these data suggest that these drugs exert their pharmacological effect at a site that is not linked with the dihydropyridine binding site.

b. CARDIAC MUSCLE. Dihydropyridine binding shows very similar characteristics in membrane preparations from cardiac and smooth muscle tissues (81, 458, 564, 953). A correlation does exist between binding affinity and potency as a negative inotropic agent for a series of nifedipine analogues (566). However, IC_{50} values in pharmacological experiments on intact cardiac tissue preparations are usually considerably higher than the affinities measured on membrane preparations. The discrepancy is illustrated in table 15, which has been constructed from papers reporting binding and pharmacological data on the same tissue. Direct electrophysiological measurements of the effect of dihydropyridines on Ca^{2+} currents have also revealed inhibitory potencies that are 2 or 3 orders of magnitude lower than expected from radioligand binding to isolated membranes (56, 609, 673, 1161).

In an attempt to resolve this discrepancy, Marsh et al. (720) investigated the binding of [^{3}H]nitrendipine to intact, cultured cardiac myocytes from embryonic chick. They detected two binding sites, a high-affinity site with a K_d of 0.2 nM and a low-affinity site with a K_d of 19 nM. They proposed that the low-affinity sites mediate the inhibitory effect (IC_{50} 23 nM) of nitrendipine on the contractile activity of the cells.

In contrast to inhibitory dihydropyridine drugs, calcium agonists are characterized by similar potencies in cardiac and smooth muscle tissues (967, 968) and by concordant affinities in binding and pharmacological experiments on cardiac tissue (see table 15). Bay K 8644 displaced [^{3}H]nitrendipine or (+)-[^{3}H]PN 200-110 binding to heart membranes with K_i values of 2 to 18 nM (672, 901, 1089). [^{3}H]Bay K 8644 bound to cultured cardiac myocytes with a K_d of 35 nM (62) and to rabbit heart membranes with a K_d of 2 to 3 nM (565). This

TABLE 15

Correlation between binding (K_d or K_i) and inotropic effect (positive, EC_{50}; negative, IC_{50}) in heart

Species	Dihydropyridine	EC_{50} (nM)	IC_{50} (nM)	K_d or K_i (nM)	Ref.
Rabbit	Nifedipine		41	1.8	(534)
Dog	Nitrendipine		1,000	0.11	(209)
Rabbit	Nicardipine		12,000	2.2	(108)
	Nifedipine		730	7.9	
Dog	Bay K 8644	6.9	4,600	30; 10,000	(1089, 1090)
	Nitrendipine		330	0.3; 140	
Guinea pig	(*S*)202-791	315		80	(1161)
	(*R*)202-791		825	0.9	
Dog	(*S*)202-791	117		230	(1161)
	(*R*)202-791		320	1.6	

binding was displaced by nifedipine and analogues with IC_{50} values that are consistent with an interaction at high-affinity binding sites. EC_{50} values for stimulation of myocardial contractility by Bay K 8644 are between 10 and 50 nM (283, 967, 1089). Bay K 8644 enhances Ca^{2+} current or ^{45}Ca uptake in cardiac myocytes with EC_{50} values of 2 to 100 nM (111, 512, 651, 901).

A pharmacological estimate of the affinity of nifedipine for its binding site can be made by measuring the pA_2 of nifedipine, using Bay K 8644 as an agonist (282, 283). Concentration-effect curves for Bay K 8644 were shifted to the right by nifedipine, and this shift was concentration dependent for nifedipine concentrations between 10 nM and 1 μM. The estimated K_d value (−log pA_2) was close to 50 nM, a value that was about one-tenth of the IC_{50} value for the negative inotropic effect of nifedipine (see also ref. 967).

These findings suggest the possibility that the high-affinity site is an "agonist" site at which antagonist dihydropyridines show insignificant intrinsic activity. As proposed by Marsh et al. (720), negative inotropic effects would then be mediated at a second, low-affinity binding site. Antagonistic effects of Bay K 8644 at micromolar concentrations have indeed been reported. Direct evidence in favor of this hypothesis has been put forward by Vaghy et al. (1090), Schwartz et al. (975), and Janis et al. (565, 566). They carried out inhibition binding experiments using rather high [^{3}H]nitrendipine concentrations to label part of the putative low-affinity sites. Inhibition curves were biphasic and compatible with a two-site model: at the high-affinity site, K_i values were 0.3 nM for nitrendipine and 6.9 nM for Bay K 8644; at the low-affinity site, K_i values were, respectively, 0.14 and 10 μM (1090) (table 15). These latter values were in good agreement with IC_{50} values (negative inotropy): 0.33 μM for nitrendipine and 4.6 μM for Bay K 8644. Moreover, nimodipine produced a very slight, but significant positive inotropic effect at concentrations of 10 to 30 nM (975). Similarly, in smooth muscle tissue (porcine coronary artery in the presence of 8.2 mM KCl), nitrendipine has been reported to have an agonistic effect at concentrations of 0.01 to 1 nM (229).

If the putative low-affinity binding site indeed mediates the negative inotropic effect of dihydropyridines, binding affinities at this site should reflect pharmacological potencies for various analogues, including stereoisomers. In contrast, Striessnig et al. (1030) have reported recently that low-affinity binding sites in cardiac membranes had inverse stereospecificity compared to high-affinity binding sites and, as in red blood cells (1029, 1031), were probably coupled to the nucleoside transporter. Moreover, B_{max} values reported for low-affinity binding sites are much higher than corresponding values for high-affinity sites (565, 1090) and greatly exceed estimates of calcium channel density provided by electrophysiological measurements on cultured cardiac cells (906) (see below).

Another interpretation of the divergence between binding and pharmacological data in heart has been advocated by Sanguinetti and Kass (951) and by Bean (57). These electrophysiologists showed that membrane potential strongly influenced the apparent potency of dihydropyridines at inhibiting Ca^{2+} current (see section III B). When cardiac cells were held at depolarized membrane potential, nitrendipine blocked currents with an IC_{50} of 0.36 nM (57). With cells held at hyperpolarized potentials, the drug was much less potent (IC_{50} 700 nM). The high-affinity binding in membrane preparations could thus reflect the inactivated state of channels in depolarized cells. This interpretation is supported by the findings of Williams et al. (1161), who used pure dihydropyridine enantiomers, (−)(*R*) 202-791 and (+)(*S*) 202-791, which act as calcium channel antagonist and agonist, respectively (table 15). The K_i for (−)(*R*)202-791 binding to isolated membranes was much lower than the IC_{50} for inhibition of contractility or calcium current measured at holding potentials of −80 or −90 mV, but correlated closely with the IC_{50} for inhibition of calcium current measured at −30 mV. Moreover, Williams et al. (1161) showed that the K_i for (+)(*S*)202-791 binding correlated with the EC_{50} for an increase in contractile force and in calcium current, this latter effect being independent of the resting holding potential.

If the lack of correlation between binding and pharmacological data as regards the action of dihydropyridine antagonists on cardiac cells arises from the "unphysiological" state of the isolated membranes used in binding studies, binding analyses carried out on intact cells under polarizing and depolarizing conditions could help resolve this discrepancy. Unfortunately, this approach is not devoid of technical difficulties. (*a*) With intact tissue, the ratio of specific to nonspecific binding tends to be lower than with purified membranes. (*b*) "Intact" cell preparations are always contaminated by a significant percentage of damaged cells. In a recent careful study, Green et al. (461) showed that [^{3}H]nitrendipine bound with the same high affinity (K_d 0.6 nM) to isolated cardiac myocytes, whether kept in normal K^+ medium or depolarized by high-K^+ or by aconitine. However, depolarization evoked a doubling of B_{max}, suggesting that it could indeed convert undetected low-affinity sites into high-affinity sites. Similar data have been obtained on skeletal muscle tissue by Schwartz et al. (977). In preliminary reports, Reuter et al. (908, 909) claimed that K^+-depolarization of living cardiac cells increased their affinities for dihydropyridines. In particular, K_i values for (*R*)202-791 and (*S*)202-791 were decreased by a factor of 20- to 40-fold. Further binding studies on intact cells are thus required to settle this point.

In studies on intact cells (208, 461), the estimated number of specific binding sites per cell (160,000 to 380,000) greatly exceeds the number of channels estimated in electrophysiological studies on cultured neonatal rat cardiac cells (2,000 to 10,000) (906). B_{max} values

for high-affinity binding determined on sarcolemmal preparations are in better agreement with electrophysiological estimates (567, 901).

In the study of Holck et al. (534) on rabbit myocardium, a good correlation was found between the abilities of phenylalkylamines and diphenylpiperazines to depress contractile responses and to inhibit [^{3}H]nifedipine binding. However, very little stereoselectivity was apparent for the effects of verapamil or D 600 isomers on binding, whereas (−)D 600 was 120-fold more potent than (+)D 600 as a negative inotropic agent. In contrast, in a more recent study (566), [^{3}H]nitrendipine binding to rabbit ventricle was reported to be 100-fold more sensitive to (−)D 600 than to (+)D 600. De Pover et al. (207) noticed that verapamil and diltiazem affected [^{3}H]nimodipine binding to canine cardiac sarcolemma and depressed contractility at similar concentrations. In addition, these authors made the striking observation that *d-cis*-diltiazem, at a concentration of 250 nM, which by itself has very little effect on contractility, strongly potentiated the negative inotropic action of nimodipine, in line with its stimulant effect on [^{3}H]nimodipine binding. Interestingly, diltiazem seems to act in the same manner as depolarization, which increases the number of high-affinity binding sites (461) and the inhibitory potency of dihydropyridines on Ca^{2+} channel current (57).

c. SKELETAL MUSCLE. The T-tubular localization of [^{3}H]dihydropyridine binding sites in skeletal muscle is consistent with the reported concentration of voltage-dependent calcium channels in that area (16). However, as in heart tissue, block of Ca^{2+} current by dihydropyridines required much higher concentrations than necessary for inhibition of binding (17). Schwartz et al. (977) have recently succeeded in measuring (+)-[^{3}H]PN 200-110 binding to intact skeletal muscle, despite the difficulties arising from the high proportion of nonspecific binding in such preparations. The same high-affinity K_d (1 nM) could be demonstrated in polarized and K^+-depolarized muscles, but B_{max} was 3 times greater in depolarized preparations. These authors compared the number of binding sites to the number of channels estimated from electrophysiological measurements. They concluded that the number of binding sites exceeds by a factor of 35 to 50 the number of channels that can be activated to pass Ca^{2+} current, which is contrary to previous estimations (387).

d. NERVOUS TISSUE. The exact significance of the binding sites for calcium channel blockers found in brain remains difficult to assess. Specific high-affinity dihydropyridine binding sites in brain are very similar to those of smooth or cardiac muscle. According to autoradiographic studies, dihydropyridine binding sites are not restricted to cerebral blood vessels, but show a distribution that suggests a preferential synaptic localization (796). However, as discussed in section II D, up to now there is only limited evidence that dihydropyridines are effective modulators of Ca^{2+} entry in neuronal preparations (excluding cultured neuronal cell lines). As suggested by Middlemiss and Spedding (751), presynaptic calcium channels may become sensitive to organic calcium antagonists only in the presence of Bay K 8644. Activation by Bay K 8644 might disappear in synaptosomal preparations (892), and this hypothesis is in line with data showing that some calcium channels lose their activity in isolated membrane patches (826) (see section III B). The discovery of various types of voltage-dependent Ca^{2+} conductances, of which only some are sensitive to dihydropyridines, may also shed light on the particular behavior of nervous tissues (see section III B).

B. Interactions with Voltage-dependent Calcium Channels

1. Structure-activity relationships and stereoselectivity. Structure-activity relationships have been extensively reviewed (e.g., 568, 715, 922, 926). Essential and nonessential structural features in the nifedipine and verapamil molecules are summarized in fig. 12.

The action of a quaternary amine derivative of verapamil (D 890) has been investigated by Hescheler et al. (511). This compound inhibits cardiac calcium channels only when applied intracellularly, probably because, being exclusively in a charged form, it is unable to reach its site of action from the outside of the cell.

The stereoselectivity of verapamil and D 600 was first examined by Bayer et al. (53, 54) on cat papillary muscle. They concluded that (+)isomers were devoid of action on calcium channels and acted only on sodium channels. However, working on the same tissue, Nawrath et al.

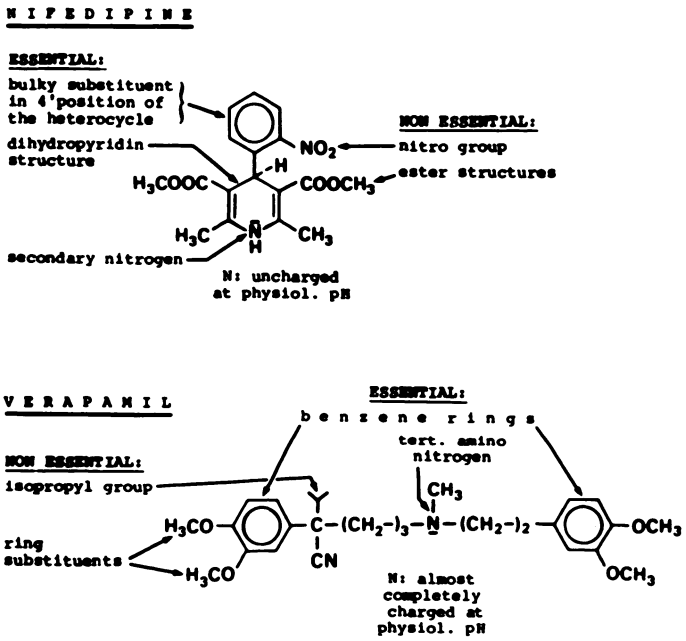

FIG. 12. Essential and nonessential features of the nifedipine and verapamil molecules with respect to their negative inotropic action in isolated heart muscle preparations. Reproduced with permission from Mannhold et al. (715).

(810) found no qualitative difference between stereoisomers. (−)Isomers were more potent than (+)isomers as negative inotropic agents, the potency ratio being 25 for verapamil and 100 for D 600. Similar results were obtained on dog heart (156), rabbit papillary muscle (534), and on human myocardium (277). According to Nawrath et al. (810), stereoisomers were equipotent as inhibitors of the maximal rate of rise of the action potential (sodium channel). The IC_{50} value for this effect was 10 μM, i.e., of the same order of magnitude as the IC_{50} values of (+)isomers on contractility (3 to 4 μM). The stereoselectivity of D 600 has been demonstrated in smooth muscle studies (577). The negative inotropic effects of other calcium channel blockers also show stereoselectivity. This has been demonstrated for prenylamine, fendiline (715), and diltiazem (207).

Small structural modifications of the nifedipine molecule give rise to compounds that can be described as calcium agonists (Bay K 8644, CGP 28392). The Bay K 8644 molecule is formed by substitution of a CF_3 group for a NO_2 group in the aromatic ring of nifedipine (see fig. 1) and of a NO_2 group at position 3 of the dihydropyridine ring instead of the ester group of nifedipine. Only the latter modification seems to be essential for the calcium agonist property. Calcium agonists were initially described as vasoconstrictor and positive inotropic agents (967, 968), but subsequently they have been shown to activate a variety of other cell types (see section II, D and E). In vascular smooth muscle preparations, Bay K 8644 has no effect on the basal tone, but it contracts tissues that are slightly depolarized by KCl (967, 968). More recently, it has been demonstrated that the calcium agonistic property is associated with only one kind of stereoisomer (343, 525, 1161).

2. Use-dependent and voltage-dependent inhibition. An important finding of electrophysiological studies is that calcium channel blockade by organic agents obeys laws similar to those of fast sodium channel blockade by local anesthetics. As shown by several workers (51, 52, 241, 647), the inhibitory effect of verapamil on the slow inward current depends upon the rate at which cardiac preparations are stimulated (use-dependent block). In cat ventricular myocardium, Ehara and Kaufmann (241) have shown that, at very low stimulation frequencies (1 to 2/min), 7 μM verapamil does not affect peak slow inward current. A lower concentration of 2 μM evokes a reduction of 30% at a frequency of 6/min, of 50% at 15/min, and of 90% at 60/min. Thus, the degree of blockade of calcium channels evoked by verapamil, D 600, and diltiazem in voltage-clamp experiments depends on the frequency of stimulation and also on the resting membrane potential, whereas these factors have been considered as less important in the action of nifedipine and other dihydropyridines (55, 474, 673, 868, 1069, 1081, 1082).

However, more recent studies have shown that blockade of Ca^{2+} channel current by dihydropyridine derivatives is modulated by membrane potential (57, 951). Block is more pronounced when Ca^{2+} channel current is measured during voltage-clamp pulses applied from depolarizing holding potentials. Correspondingly, in preparations that are not voltage clamped, blocking activity of these compounds is strongly influenced by cell resting potential. Sanguinetti and Kass (951) have proposed that the modulated receptor hypothesis that explains block of Na^+ channels by local anesthetics in nerve and skeletal muscle (519) could be applied to Ca^{2+} channel blockade by calcium antagonists. This theory proposes that binding of a drug to a site located within the channel is influenced by the state of the channel and that this state is determined by membrane potential. This model predicts that ionized drugs can only gain access to the channel-associated binding site via a hydrophilic pathway that is available only when channels are in an open state configuration. Neutral drugs can reach this site through this pathway and by a hydrophobic route through the lipid membrane surrounding the channel. Verapamil is almost entirely in a charged form at pH 7.4, whereas nifedipine, nisoldipine, and nitrendipine are neutral. Assuming that, in depolarized membrane, calcium channels may be in a resting (R), open (O), or inactivated (I) state, the sequence of channel states in the presence of verapamil (V) would be the following: R → O → OV → IV. For verapamil and related drugs, block is frequency dependent because repetitive depolarizing stimuli increase the probability for the channel to be in an open state.

Neutral dihydropyridines (D) may reach their site of interaction via two pathways: R → O → OD → ID or R → I → ID. Thus, for these drugs, the frequency of stimulation is less important, but block is voltage dependent, because the second pathway of interaction is possible at depolarized holding potentials, which increase the proportion of inactivated channels, in the absence of repetitive stimulation. In agreement with the modulated receptor hypothesis, Sanguinetti and Kass (951) found some degree of use-dependent block with nicardipine, a partly charged dihydropyridine.

In contrast with the hypothesis described above, Kanaya et al. (599) proposed that diltiazem and verapamil preferentially bind to inactivated cardiac calcium channels.

As mentioned in section III A, preferential binding of dihydropyridines to the inactivated state of the channel may account for the 1000-fold difference in potency when contractility or electrophysiological data obtained on intact cardiac preparations are compared to binding data obtained on isolated membranes. Bean (57) found that, when cardiac cells were held at hyperpolarized potentials, nitrendipine blocked Ca^{2+} currents with an apparent K_d of 700 nM, whereas when cells were held at depolarized membrane potentials at which Ca^{2+} currents were 70% inactivated, nitrendipine blocked Ca^{2+} currents much more potently, with an apparent K_d of 0.36 nM, a value

very similar to that found in binding studies on isolated, and presumably depolarized, membranes.

The voltage dependency of dihydropyridine action might explain why these drugs are more potent on vascular smooth muscle than on cardiac muscle. Indeed, in vascular smooth muscle preparations, resting membrane potential may be more positive than in cardiac cells. Moreover, the inhibitory potency of these drugs on K^+-depolarized smooth muscle preparations may be expected to be very high.

In studies on arterial smooth muscle, Godfraind and coworkers (408) found that the rate of ^{45}Ca entry decreased with the duration of K^+-depolarization, suggesting progressive inactivation of calcium channels. In contractility and ^{45}Ca flux studies, calcium channel block by flunarizine, nisoldipine, and nimodipine (and, to a lesser degree, nifedipine) increased with the duration of depolarization (see section II A 1). Thus, these drugs may interact preferentially with the inactivated state of voltage-dependent channels, and they may have less affinity for open channels. Similarly, in rat uterine tissue, D 600 was more potent as an inhibitor in K^+-depolarized than in polarized preparations (768).

Bolton et al. (88) reported use-dependent effects of calcium entry blockers on the electrical and mechanical activities of guinea pig *taenia caeci.* Interestingly, qualitatively similar interaction patterns were observed with nifedipine, diltiazem, and D 600.

3. Subtypes of voltage-dependent calcium channels and the mode of action of dihydropyridines as revealed by patch clamp studies. Thanks to the recent development of patch clamp analysis, ionic currents flowing through single Ca^{2+} channels have been recorded in cultured cardiac, chromaffin, and neuronal cells. The basic properties of Ca^{2+} channels seem to be similar in all these tissues. As reported by Reuter (907), upon depolarization, the opening of channels frequently occurs in bursts. With long depolarization clamp steps (1 s), the frequency of openings decreases during the pulse, indicating inactivation of the channels. Inactivation is partially voltage dependent and results from a decreased probability of Ca^{2+} channel opening when the holding potential is made more positive. In cardiac preparations, the probability of channels entering an open state during membrane depolarization is likely to be regulated by cyclic AMP-dependent phosphorylation of the Ca^{2+} channels or of a protein closely associated with the channel (906). It seems that Ca^{2+} channels need some input from cell metabolism for their proper functioning. In contrast to Na^+ channels, they do not survive in isolated inside/out membrane patches.

Hess et al. (512) and Kokubun and Reuter (651) have studied the interaction of dihydropyridines with cardiac Ca^{2+} channels. From their results, it may be concluded that the inhibitory action of calcium antagonists on these channels cannot be explained on the basis of a simple plugging of the channels. They have reported the existence of different modes of Ca^{2+} channel gating favored by dihydropyridine Ca^{2+} agonists and antagonists. In the absence of drugs, they have characterized three modes of gating behavior, expressed as current records with brief openings (mode 1), with no opening (mode 0 or null mode), and with long-lasting openings and brief closing periods that appear only rarely (mode 2). Mode 0 is favored by nitrendipine and nimodipine, while Bay K 8644 enhances Ca^{2+} channel current by promoting mode 2. This modulation by dihydropyridines of the gating of Ca^{2+} channels does not appear to be related to cyclic AMP-dependent phosphorylation.

Recently, it has become apparent that there are distinct subtypes of potential-dependent Ca^{2+} channels in neuronal cells (94, 126, 127). Tsien and coworkers have reported distinct types of Ca^{2+} channels in cultured sensory neurones of the chick dorsal root ganglion (835) and in mammalian cardiac cells (826). In cardiac cells, they have found two types of unitary conductance with different kinetic features, which can be attributed to the opening of two distinct Ca^{2+} channels. The most common, observed in many cells, has been called the L-type. Repeated opening of these produces a long-lasting inward Ca^{2+} current through the membrane. A second type of channel, termed the T-type, opens at much more negative membrane potentials than the L-type and produces a transient inward membrane current. In chick dorsal root ganglion cells, a third type of conductance (N-type) has been detected by Nowycky et al. (835). N-type channels require strongly negative potentials for complete removal of inactivation (unlike L) and strong depolarizations for activation (unlike T), and they inactivate relatively rapidly.

In addition to the different potential ranges at which they are gated, the channel subtypes can be distinguished by different sensitivities to pharmacological agents. In cardiac and neuronal cells, only L-type channels are influenced by dihydropyridines, including Bay K 8644 (826, 834, 835). In cardiac cells, nimodipine (5 μM) halved the average current through the L-type Ca^{2+} channel, but the same concentration failed to reduce T-channel activity. It is likely that the sensitivity of a given tissue to dihydropyridines will ultimately depend upon the respective proportion of various Ca^{2+} channel subtypes.

Single Ca^{2+} channel currents have recently been recorded in isolated smooth muscle cells from guinea pig *taenia coli* (1182). Thus, it may be expected that further electrophysiological analyses will examine the possible existence of several subtypes of voltage-dependent calcium channels in smooth muscle (see ref. 543 and section III F).

C. Interactions with Calmodulin

Phenothiazines (chlorpromazine and trifluoperazine), the butyrophenone, haloperidol (678–682), and a series of naphthalenesulfonamides (516–518) were the first compounds demonstrated to antagonize the activity of

calmodulin (reviewed in ref. 1143). These compounds inhibit contractions in isolated rabbit aorta elicited by agonists and by depolarization (597).

However, it has been known almost since the concept of calcium antagonism was first discussed that chlorpromazine exerts actions that are in some respects similar to cinnarizine, i.e., inhibits Ca^{2+} entry into cells (287, 435, 984), and recently it has been shown that other phenothiazines (287) and the naphthalenesulfonamide, W-7 (499, 500), are also able to inhibit ^{45}Ca influx into cells. Trifluoperazine and other calmodulin antagonists weakly antagonize action potentials in ventricular cells, and this effect has been attributed to inhibition of Ca^{2+} influx (582). Trifluoperazine also inhibits ^{45}Ca influx stimulated by carbamylcholine in adrenal medullary cells (1122). These observations all tend to show that inhibition of Ca^{2+} influx is associated with antagonism of calmodulin.

In acellular test systems, an interaction of some calcium entry blockers with calmodulin, that resembles that of calmodulin antagonists, has been demonstrated. Diphenylalkylamines such as bepridil, cinnarizine, flunarizine, and the dihydropyridines, felodipine and nicardipine, bind to calmodulin and inhibit enzyme activation by calmodulin (95, 561, 580, 701, 990). The binding of the dihydropyridine, felodipine, to calmodulin was allosterically modulated by D 600 and diltiazem (579, 582), in a manner analogous to dihydropyridine binding to calcium channels (209, 275). Furthermore, Luchowski et al. (699) showed that the affinity of calmodulin antagonists for nitrendipine binding sites correlates well with their binding to calmodulin.

Considering these coincidences of effects between calmodulin antagonists and calcium entry blockers, the hypothesis has been put forward that a regulatory protein in, or associated with, Ca^{2+} channels might have similar binding properties to calmodulin (579, 581, 699).

However, verapamil and diltiazem, as well as most dihydropyridine derivatives, have a very low affinity for calmodulin and do not inhibit contraction in skinned smooth muscle fibers (562, 657, 749, 1010), and the effectiveness of many dihydropyridines as inhibitors of purified phosphodiesterases is generally about 3 orders of magnitude less than their effectiveness at displacing [^{3}H]nitrendipine binding (1099). Compounds such as diltiazem, bepridil, cinnarizine, and its difluoro derivative, flunarizine, interact with calmodulin in a concentration range (10 to 200 μM) that is at least an order of magnitude greater than their active concentrations as calcium entry blockers in vascular tissue (10 nM to 3 μM; section II A). IC_{50} values for cinnarizine, for example, are in the region of 40 nM for inhibition of depolarization-induced contractile responses in vascular tissues, about 60 μM for inhibition of superprecipitation of arterial actomyosin and phosphorylation of myosin light chain (990), and about 10 μM for inhibition of phosphodiesterase (701). Diltiazem at a concentration of 80 μM binds to calmodulin (580), but it inhibits depolarization-induced contractions of vascular tissue in the 0.2 to 1 μM range (section II A). Also, in skinned smooth muscle preparations, an antagonism of Ca^{2+}-induced contractions by fendiline, cinnarizine, and flunarizine has been reported (1010), but active concentrations were high (100 μM range). In normal vascular preparations such as rat and rabbit aorta, none of the above-mentioned calcium entry blockers affects markedly the efflux of ^{45}Ca, a process known to be dependent on calmodulin (section II A), at concentrations (up to 3 μM) that abolish ^{45}Ca influx (157, 410, 428, 1092). Furthermore, calcium entry blockers do not inhibit contractions mediated by the release of intracellular Ca^{2+} stores (section II A; table 2), even though some of them, such as bepridil and cinnarizine (179a, 433, 783, 854a), appear to be concentrated in cells to an extent severalfold ($\geq$ 10) that of the nominal extracellular concentration. It therefore seems that calcium entry blockers do not normally exert effects associated with antagonism of calmodulin in whole cells, and it is questionable if calmodulin itself constitutes part of the calcium channel and a receptor for some calcium entry blockers.

D. Interactions with Alpha-Adrenoceptors

It has been known for many years that high concentrations (in the region of 0.1 mM) of the irreversible alpha-adrenoceptor antagonists, dibenamine and phenoxybenzamine, can inhibit contractions of rabbit aorta (70, 71, 983) and guinea pig *taenia coli* (984) induced in solutions containing high concentrations of K^+. Inhibition of such contractions has now become almost enough to define a compound as a calcium antagonist ("Introduction") and could indicate an ability to inhibit depolarization-induced influx of extracellular calcium into the smooth muscle cells, as was demonstrated by Shibata and colleagues (984) who suggested that the effects of chloropromazine, phenoxybenzamine, and dibenamine were mediated by an inhibition of calcium movement. It is, therefore, not surprising that more recently some thought has been given to the possibility of an interaction between alpha-adrenoceptors and calcium channels, and phenoxybenzamine and related compounds have been much studied for their possible calcium antagonist effects.

K^+ (80 mM, 1.8 mM Ca^{2+})-induced contractions of guinea pig ileum were inhibited 50% by about 62 μM phenoxybenzamine, K^+-induced concentration-effect curves were displaced to the right, and the maximal responses were inhibited by phenoxybenzamine (10 nM to 50 μM) (374). In this tissue, [^{3}H]nitrendipine binding was irreversibly inhibited by phenoxybenzamine (IC_{50} 5.1 μM). Scatchard analysis showed that phenoxybenzamine reduced the K_d for [^{3}H]nitrendipine binding without effect on B_{max} except at the highest concentration

tested, 0.1 mM (374). Preincubation with 30 μM of the compound SKF 525A, that has calcium entry blocking activity (see section II C), did not protect against blockade of norepinephrine-induced contractions in rat vas deferens by subsequent exposure to phenoxybenzamine (1040), but preincubation with 10 μM D 600 did provide a measure of protection (836). The effect of phenoxybenzamine on the binding of nitrendipine has been interpreted as an allosteric effect (374).

In guinea pig *taenia caeci* relatively high concentrations of the calcium entry blockers D 600 (0.5 μM), diltiazem (10 μM), and nicardipine (30 nM) were reported to potentiate the inhibitory effects of phenoxybenzamine (measured as inhibition of histamine-induced contractions), but slightly lower concentrations of the blockers (0.1 μM, 3 μM, and 3 nM, respectively) were without effect on the action of phenoxybenzamine (1085). K^+ (40 mM) responses of guinea pig *taenia caeci* were not affected by a combination of calcium entry blocker and phenoxybenzamine to a greater extent than by the blocker alone (1085). This apparent lack of additive effects that might be expected between antagonists is not uncommon between calcium entry blockers (see section II B).

Antagonism of muscarinic-receptor mediated responses in mouse neuroblastoma cells by phenoxybenzamine and dibenamine has been attributed to an interaction between the antagonists and calcium channels (245). With these cells, the antagonism by phenoxybenzamine and dibenamine of the increase in cyclic GMP evoked by muscarinic-receptor stimulation, but not the binding of the antagonists to muscarinic receptors, was antagonized by increasing the Ca^{2+} concentration.

In an extensive comparison of the antagonist effects of phenoxybenzamine in rabbit aorta, McPherson et al. (737) found that a concentration (0.5 μM) that inhibited norepinephrine contractions did not affect histamine-induced responses. Concentrations of phenoxybenzamine sufficient to inhibit histamine contractions (1 to 5 μM) also inhibited Ca^{2+}-induced contractions of K^+-depolarized preparations. In rat aorta, norepinephrine-induced responses were almost abolished by low concentrations of phenoxybenzamine (0.05 μM), but a concentration of 0.5 mM was needed to partially antagonize Ca^{2+}-induced contractions. Contractions of guinea pig ileum preparations induced by carbachol and histamine were much more sensitive (at least 15-fold) to antagonism by phenoxybenzamine than were Ca^{2+}-induced contractions. Norepinephrine contractions of guinea pig spleen were depressed by 10 nM to 1 μM phenoxybenzamine but, as in rat aorta, 0.5 mM was required to antagonize Ca^{2+} contractions. These authors also concluded that phenoxybenzamine may inhibit agonist-induced contractions by interaction with receptor-operated calcium channels (737). Assuming that phenoxybenzamine inhibition of Ca^{2+} contractions is due to interaction with Ca^{2+} channels, their results would indicate marked differences between the potential-dependent channels of rabbit aorta and those of guinea pig spleen and rat aorta. As far as the aortic tissues are concerned, such differences are not apparent with the more usual calcium entry blockers (table 2; section II A 2).

The study of possible calcium antagonist properties of alpha-adrenoceptor antagonists unrelated to phenoxybenzamine has also provided interesting data. Yohimbine at very high concentrations (432) and the two yohimbine stereoisomers, rauwolscine and corynanthine (pA_2 values variously reported as between 6 and 7.5 in rat aorta), also inhibit K^+-induced contractions of rat aorta (IC_{50} values of about 30 μM), and this inhibition was correlated with an inhibition of ^{45}Ca influx (432). Furthermore, the $alpha_1$-adrenoceptor selective antagonist, nicergoline (pA_2 about 8.6 in rat aorta), antagonized depolarization-induced ^{45}Ca influx in rat aorta and inhibited K^+-contractions (IC_{50} about 30 μM) (496). The irreversible alpha-adrenoceptor antagonist, benextramine, and the competitive antagonist, WB 4101, both of which are also selective for $alpha_1$-adrenoceptors in rat brain membranes, inhibited K^+-stimulated ^{45}Ca uptake by rat brain synaptosomes with IC_{50} values of 10 ± 5 μM and 90 ± 5 μM, respectively, and in this regard were more potent than D 600, verapamil, and nicardipine (876). Benextramine antagonism of ^{45}Ca uptake was reversible, indicating a lack of identity of calcium channels and alpha-adrenoceptors.

In neuroblastoma-glioma hybrid cells (NG 108-15), Ca^{2+}-dependent spikes were inhibited by WB 4101 (IC_{50} 48 μM) and D 600 (IC_{50} 80 μM) (34). The electrically stimulated Ca^{2+}-dependent release of GABA from slices of cortex has been shown to be antagonized by yohimbine, rauwolscine, corynanthine, phentolamine, RS 21361, and idazoxan, all at concentrations of about 1 to 10 μM, but not by pseudoyohimbine or prazosin, by a mechanism independent of blockade of $alpha_2$-adrenoceptors (732). The common mechanism of action might be related to a blockade of calcium entry. In nonnervous tissues, neither prazosin nor phentolamine has an inhibitory effect on K^+-induced responses that cannot be attributed to antagonism of the effects of norepinephrine liberated from nerve terminals. In vascular tissue, such as rat aorta, that has little or no innervation (863), neither of these alpha-adrenoceptor antagonists affects K^+-induced contractions.

On the other hand, some calcium entry blockers have been shown to interact with alpha-adrenoceptor binding sites. In rat brain membranes, verapamil, D 600 and nicardipine (YC-93) displaced the $alpha_1$-adrenoceptor ligands [^{3}H]WB 4101 (K_i values, respectively, 0.31, 0.93, and 4 μM) and [^{3}H]prazosin (K_i values of 1.7, 1.1, and 3.2 μM, respectively) (34, 876). In another report, the K_i for D 600 displacing [^{3}H]prazosin was 2.8 μM (391). Displacement of [^{3}H]dihydroergocryptine binding to $alpha_1$-adrenoceptors of rabbit uteri has been demon-

strated for cinnarizine (IC_{50} 7 μM), flunarizine (25 μM), and verapamil (2.5 μM) (463), while nifedipine, nimodipine, and felodipine were inactive (666). In rat vas deferens, the K_i value for D 600 inhibition of [^{3}H]WB 4101 binding was about 2 μM but, unlike the calcium entry blocking action of D 600, this was nonstereoselective (577). Nonstereoselective displacement by verapamil (K_i about 5.8 μM) of [^{3}H]prazosin binding to rat cardiac membranes has also been demonstrated, and (±)D 600 (K_i 6.31 μM), diltiazem (165 μM), and nicardipine (9.25 μM) but not nifedipine also displaced [^{3}H]prazosin (819). Rather smaller K_i values for verapamil (0.6 μM) and D 600 (0.34 μM) in this tissue were obtained by others (refs. 607 and 391, respectively). Verapamil (K_i 0.1 μM), but not nifedipine or diltiazem, inhibited [^{3}H]dihydroergocryptine binding to rabbit urethra (23). Verapamil displaced [^{3}H]prazosin binding from rat renal cortex (K_i 2 μM) and from BC3H-1 smooth muscle-derived cells (K_i 1 μM) (780). Calcium entry blockers displaced [^{3}H]prazosin specific binding from rat aortic smooth muscle membranes according to the rank order of potency: flunarizine (K_i 1.9 μM) > cinnarizine (K_i 2.9 μM) = verapamil (K_i 2.9 μM) > bepridil (K_i 9.2 μM) ≫ diltiazem (K_i 70 μM). Nifedipine and Bay K 8644 were inactive (211).

In rat brain membranes, the K_i values for displacement of an $alpha_2$-ligand [^{3}H]clonidine were: verapamil (15 μM); D 600 (≫100 μM); and nicardipine (>100 μM) (34, 876). Both verapamil and D 600 displaced [^{3}H]yohimbine binding (to $alpha_2$-receptors) with K_i values of 2 and 10 μM, respectively (353). [^{3}H]Clonidine binding was displaced by D 600 [K_i 9.3 μM (391); 13.8 μM (819)], diltiazem (15.2 μM), nicardipine (19.7 μM), and stereoselectively by verapamil [(+)verapamil (K_i 13.8 μM), (−)verapamil (2.2 μM)], while nifedipine was inactive (819). [^{3}H]Yohimbine binding to human platelet membranes was displaced by verapamil (K_i 1.6 μM), D 600 (2.2 μM), fendiline (2.6 μM), and prenylamine (1.6 μM), but not by diltiazem, nitrendipine, or nifedipine. Binding to rat renal cortex was displaced by verapamil (K_i 5.6 μM) (780). As was the case with $alpha_1$-adrenoceptors, verapamil, diltiazem, flunarizine, cinnarizine, and bepridil, but not nifedipine or Bay K 8644, displaced [^{3}H]yohimbine specific binding from rat aortic smooth muscle membranes. Diltiazem was again less potent (K_i 70 μM) than verapamil (6.8 μM), flunarizine (3.3 μM), cinnarizine (3.4 μM), and bepridil (17.5 μM) (211).

A consistent finding from laboratories where interactions with both $alpha_1$- and $alpha_2$-adrenoceptor binding sites were studied was that verapamil, D 600, cinnarizine, flunarizine, and bepridil preferentially interacted with $alpha_1$-adrenoceptor binding sites. Nicardipine was almost inactive on $alpha_2$-adrenoceptor binding sites in rat brain membranes, although it displaced [^{3}H]prazosin binding (K_i about 3 μM), but in rat heart membranes, it was only about 2-fold more potent at $alpha_1$- than at $alpha_2$-sites. Diltiazem was either equipotent (rat aorta) or more potent at $alpha_2$-sites (rat heart). As far as $alpha_1$-adrenoceptor sites are concerned, verapamil, D 600, diltiazem, and the dihydropyridines displayed about the same degree of affinity (or had a lack thereof) in the various tissues studied. Of the active compounds, concentrations of nicardipine interacting with alpha-adrenoceptors (K_i in the μM range) are considerably higher than those usually associated with calcium entry blockade in vascular smooth muscle. But verapamil, D 600, bepridil, flunarizine, and cinnarizine displaced radiolabelled alpha-adrenoceptor antagonists from their receptor at concentrations of about the same magnitude, or only slightly greater than the concentrations that abolish calcium entry in peripheral tissues, and the possible importance of this interaction should be taken into account in the design of experiments. However, it should be noted that, in contractility studies, these same calcium entry blockers do not displace norepinephrine concentration-effect curves to the right to a marked extent, as might be expected of an alpha-adrenoceptor antagonist, but, for example, display a noncompetitive type of inhibition in rat aorta and have only minor inhibitory effects in rabbit aorta preparations even at high concentrations (section II A). Therefore, alpha-adrenoceptor antagonistic effects of calcium entry blockers, used at reasonable concentrations, are probably not a major complicating effect in the interpretation of experimental data.

The overall number of observations showing a relationship between alpha-adrenoceptors and calcium channels is impressive and, while the possibility of overlap between the two entities has been raised, it probably indicates that the coupling between these two structures in the membrane is such that a change in conformation of one affects the other.

E. Other Sites of Interaction

Occasionally, other possible sites of action of calcium channel blockers have been suggested in studies on isolated membranes and organelles. However, it should be borne in mind that the only established distinguishing feature of this class of therapeutic agents is their ability to reduce Ca^{2+} influx into cells subsequent to a physiological or pathological stimulus. The significance of additional actions often remains doubtful, but some of them, especially those that have been observed at reasonable concentrations, may contribute to the pharmacological profile of particular drugs.

1. Plasma membrane. a. Na^+-Ca^{2+} EXCHANGE. Takeo et al. (1050) reported that nicardipine depressed Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles. The initial rate of ^{45}Ca uptake by Na^+-loaded vesicles was reduced by 60% by 0.1 μM nicardipine. As Na^+-Ca^{2+} exchange is an important mechanism of Ca^{2+} extrusion in cardiac cells, inhibition of this mechanism by nicardipine might counteract somewhat the negative inotropic effect of this drug. This might explain why nicardipine is considerably less potent than nifedipine as a negative inotropic agent, although it has more affinity for calcium

channels (108). Verapamil inhibited Na^+-Ca^{2+} exchange in synaptic membranes only at high concentrations (IC_{50} 175 μM) (254).

b. ATP-DEPENDENT Ca^{2+} TRANSPORT. Nifedipine (but not D 600) has been reported to stimulate ATP-dependent Ca^{2+} transport in cardiac sarcolemmal vesicles (presumably inside-out) from very young SHR (192). Stimulation amounted to 50 to 60% at concentrations of 0.1 to 1 μM. In membranes from control rats, stimulatory effects required higher concentrations (10 to 100 μM). The authors suggested that enhanced Ca^{2+} extrusion from cardiac cells might be related to the antihypertensive action of nifedipine in SHR. A stimulatory action of nitrendipine on Ca^{2+} pumping out of vascular smooth muscle cells has also been postulated by Hermsmeyer (510).

Verapamil (1 μM) has been shown to inhibit passive Ca^{2+} binding to cardiac sarcolemma and its ATP-dependent Ca^{2+} transport capacity (726). The activity on the Ca^{2+} transport ATPase resided in the (−)isomer.

c. Na^+,K^+-ATPase. Nimodipine (1.5 nM to 1 μM) increased Na^+,K^+-ATPase activity by 50 to 120% in membranes isolated from aorta and vas deferens, but not in membranes from heart or brain (853). Nitrendipine had less effect, and nifedipine, verapamil, and diltiazem were inactive. It is not known whether this effect of nimodipine, which occurs at pharmacologically relevant concentrations, could contribute to its relaxant action in intact smooth muscle tissue.

d. NUCLEOSIDE TRANSPORTER. As mentioned in section II F, [^{3}H]nimodipine specific binding sites in erythrocytes have been identified as the nucleoside transporter. In dog heart and brain, dihydropyridines have been shown to compete with the binding of [^{3}H]nitrobenzylthioinosine, a potent inhibitor of adenosine uptake (716). K_i values of 20 to 40 nM were obtained with nimodipine, the most potent dihydropyridine.

e. OTHER PLASMA MEMBRANE SITES. Verapamil and D 600 inhibit the uptake of serotonin, dopamine, and norepinephrine by synaptosomes with IC_{50} values of 2 to 30 μM (736). They displace [^{3}H]phencyclidine binding in rat brain membranes (K_i 1 to 2 μM) (889). Their antagonistic effects at Na^+ channels and alpha-adrenoceptors have been discussed previously.

2. *Sarcoplasmic reticulum and mitochondria.* Calcium entry blockers may influence Ca^{2+} sequestration by skeletal and cardiac sarcoplasmic reticulum vesicles (170, 1057, 1126), but these effects usually required excessively high concentrations of these drugs. However, at a concentration of 3 μM, diltiazem blocked Ca^{2+} release from cardiac sarcoplasmic reticulum vesicles evoked by changing K^+ to $Tris^+$ in the suspension medium (cationic "depolarization") (522). This effect of diltiazem might be related to its reported capacity to inhibit Ca^{2+} mobilization from intracellular (sarcoplasmic reticulum) stores in vascular smooth muscle (557, 944, 945).

Diltiazem inhibits Na^+-induced Ca^{2+} release from heart and brain mitochondria (728). The IC_{50} value is much lower for *d-cis*-diltiazem (7 μM) than for *l-cis*-diltiazem (350 μM). At high concentration, diltiazem and other drugs inhibit phosphate-induced swelling of heart mitochondria (729).

F. Tissue Selectivity of Calcium Entry Blockers

One of the most important and interesting questions is: does tissue selectivity exist, and if so, to what properties of the antagonists and tissues is it due? Some aspects of this question have been discussed previously [e.g., Cauvin et al. (132); Godfraind (400); Janis and Triggle (568); Kazda et al. (621)].

1. Mechanisms of tissue selectivity. As far as the cardiovascular system is concerned, agents that depress cardiac contractility are not generally sought after by clinicians, and a primary concern is the relative selectivity of the antagonists for vascular tissue over cardiac tissue. Fig. 13 confirms that the vascular selectivity of dihydropyridines can be demonstrated in isolated preparations from human heart tissue and coronary arteries (413). Furthermore, it was observed that different dihydropyridines differed by their relative potencies in these human preparations. For instance, nisoldipine had an activity 10 times higher than nifedipine in coronary arteries, but 10 times lower in cardiac tissue (Godfraind et al., unpublished). Thus, nisoldipine appears 100 times more specific for human coronary arteries than nifedipine. The selectivity of action, or lack of it, for blood vessels from different regions (623, 1065) is an equally interesting possibility, offering as it does the potential to alter perfusion regionally. For instance, Godfraind and Miller (428) compared the inhibitory action of three calcium entry blockers in K^+-depolarized preparations from rat aorta and human coronary arteries. The order of potency was nifedipine > flunarizine > lidoflazine in rat aorta and nifedipine > lidoflazine > flunarizine in human coronary arteries.

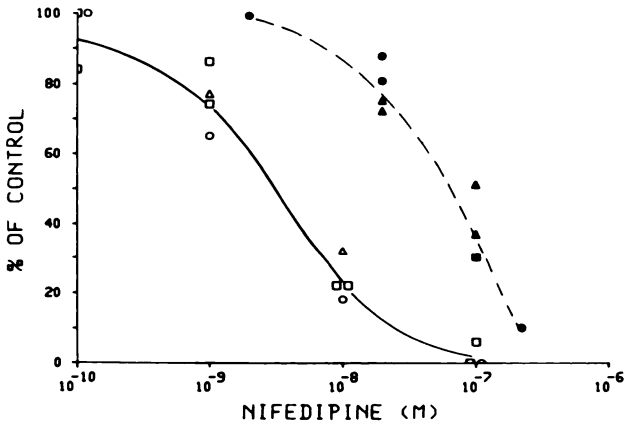

FIG. 13. Concentration-effect curves of nifedipine in human isolated coronary artery segments contracted by depolarization (*solid line*) and electrically stimulated trabecular muscle (*broken line*) taken from the same hearts aged 45 (○, ●), 43 (□, ■), and 15 (△, ▲) yr. Reproduced from Godfraind et al. (413).

The general indiscriminate use of "calcium antagonist" as a description of compounds that may not, in fact, share exactly the same mechanism of action has led to many misunderstandings. For the group of calcium entry blocking compounds, there are, considering a single species, three fundamental factors that may account for a selectivity of action.

a. Calcium entry blockers, as the name implies, can only be effective inhibitors if activation of the tissue is dependent to a significant extent on entry of extracellular calcium. Myocardium, for example, as has been known for many years, is dependent to a very large extent on extracellular calcium to sustain contractility. Therefore, a calcium entry blocker might be expected to inhibit cardiac contractions. In vascular smooth muscle, the picture is less well defined because, in many vessels, contractions can be induced in the absence of extracellular calcium (see section II A). In other tissues, the dependence or otherwise of the response being studied on extracellular calcium is also quite variable. In endocrine tissues (e.g., isolated glomerulosa cells and pituitary cells) and intestinal smooth muscle, the potency of dihydropyridines as inhibitors of responses to "physiological" stimuli is distinctly lower than their potency as inhibitors of K^+-depolarization-evoked responses and, in the case of endocrine tissues, it has been demonstrated that the difference is due to the ability of agonists (angiotensin II and TRH, respectively) to mobilize intracellular calcium stores (see section II E). In other cell types (platelets, mast cells, neutrophils, skeletal muscle, exocrine pancreas, respiratory smooth muscle), the dependence on extracellular Ca^{2+} for activation appears doubtful, and depressing effects of calcium entry blockers have generally been observed only at high concentrations at which nonspecific effects are likely (see section II, B, F, and G). Even if responses are dependent on extracellular Ca^{2+}, there is always the possibility that Ca^{2+} entry into the cytoplasm is affected by a mechanism other than a specific channel, for example, via an ion exchanger (see also section II, A 2 and D 2).

Compounds that exert "calcium antagonist" effects at an intracellular level and that are effective inhibitors of stimulus-evoked responses, for example, agents that alter the affinity of calmodulin for Ca^{2+}, would be expected to antagonize those responses whether they were dependent on intracellular or extracellular calcium or both.

b. Calcium channels probably do not constitute a single homogenous population in different tissues (478) or even within a given tissue (94, 530, 543, 826, 835). In consequence, the affinity of different calcium channels for different blockers may vary (see below). If this was true, regional differences in the same tissue could exist, as well as differences between tissues.

c. The conditions under which voltage-dependent calcium channels are activated play a major role in tissue selectivity. Indeed, the duration and/or frequency of stimulation, as well as the resting membrane potential, will markedly influence the potency of some calcium entry blockers (see section III B 2). For example, while dihydropyridines do inhibit calcium channels in cardiac tissue, their potency on electrically stimulated tissues is comparatively low (see, for example, fig. 13), probably because, in these preparations, the time spent by the channels in an inactivated state is too short to allow dihydropyridines to bind at low concentrations (57).

The first factor (a) can and should be taken into account, but the influence of the other factors (b) and (c) is not at present known with any certainty. The possibility of differences between the same tissue of different species as regards the above points has the potential to further complicate the interpretation and generalization of experimental results.

2. *Agonist-dependent selectivity.* Another important consideration, a direct consequence of (a) discussed above, arises when considering agonist stimulation of contractile and other responses. If different agonists have differing abilities to mobilize intracellular and extracellular calcium in the same tissue, there will be an apparent selectivity for inhibition of contraction induced by one or other agonist, that is not a property of either the calcium channel (assuming a channel to be involved) or the calcium entry blocker in question. Further, the properties of a single agonist in different tissues should not be overlooked. For example, in the case of alpha-adrenoceptor agonists, it was first shown by Van Meel et al. (1100) that, in rat in vivo (pithed) preparations, the alpha$_2$-adrenoceptor selective agonist B-HT 920-stimulated increases in blood pressure were more susceptible to antagonism by the calcium entry blockers nifedipine, D 600, and verapamil than were responses induced by the alpha$_1$-adrenoceptor selective agonist, methoxamine. It was, therefore, proposed that alpha$_2$-adrenoceptor agonists were more dependent on extracellular Ca^{2+} to produce contractile effects than alpha$_1$-adrenoceptor selective agonists. This observation was confirmed in vivo using a variety of agonists and calcium entry blockers (138, 777) and in vitro in isolated arterial preparations of rat using cinnarizine and D 600 (431, 822) and in the perfused dog hindlimb using verapamil (691), although in this latter preparation, there was no differential antagonist effect of cinnarizine. Also, in the dog saphenous vein, it was found that responses induced by the alpha$_1$-adrenoceptor selective agonist, methoxamine, were inhibited to a greater extent by verapamil than were responses to the alpha$_2$-adrenoceptor selective agonist, clonidine (205), and Janssens and Verhaeghe (570) reported that, in this tissue, the alpha$_2$-selective agonists, clonidine and guanfacine, induced both Ca^{2+} influx and release of intracellular Ca^{2+}.

These divergent results raised the possibility that the subtypes of alpha-adrenoceptors were not linked to mechanisms activating calcium influx and intracellular

calcium release in an identical manner in all situations. However, an investigation of the ability of several alpha$_2$-selective agonists to release intracellular calcium in the dog saphenous vein has convincingly demonstrated that responses to alpha$_2$-adrenoceptor agonists are essentially mediated by Ca^{2+} influx, liberation of intracellular Ca^{2+} being attributable to stimulation of alpha$_1$-receptors (576). The observations of De Mey and Vanhoutte (205) might be explained by an alpha-adrenoceptor blocking action (alpha$_1$-selective) of verapamil (see section III D) (K_i values in the range 0.6 to 5 μM).

Many alpha$_2$-adrenoceptor agonists, including those mentioned above, are considered to be partial agonists. A partial agonist is one that usually elicits a smaller maximum response than some reference compound (a full agonist and usually the neuromediator in the tissue in question) and has a smaller efficacy and therefore no spare receptors (625). Since alpha$_2$-adrenoceptor selective agonists are relatively more dependent on extracellular calcium than are alpha$_1$-adrenoceptor agonists (of which most are full agonists) to produce a contractile response, there is therefore an apparent link between efficacy and ability to activate intracellular calcium in vascular smooth muscle. It has been shown that in vivo a receptor reserve exists for alpha$_1$- but not for alpha$_2$-adrenoceptors (481, 898, 938). After the removal of the receptor reserve for cirazoline (937) or methoxamine (865) by pretreatment with phenoxybenzamine, the alpha$_1$-agonist-stimulated increases in blood pressure are inhibited by diltiazem in a similar manner to B-HT 920-induced responses. A similar result has been found in vitro using a variety of alpha-adrenergic agonists (172, 575). A comparison of a series of alpha$_1$-adrenoceptor selective agonists in dog saphenous vein has shown that the ability of nifedipine to antagonize contractions was inversely related to the intrinsic activity of the agonists (575).

Extended in vivo experiments, utilizing a variety of alpha-adrenoceptor agonists, have shown that the ability of nifedipine, nimodipine, and verapamil to inhibit pressor responses varies with the agonist and not necessarily with its receptor selectivity (1059, 1060). Notably, the alpha$_1$-adrenoceptor selective agonist indanidine (Sgd 101/75) (1135) is inhibited by nifedipine in an identical manner to B-HT 920, and in the experimental conditions, there were no, or very few, spare receptors for Sgd 101/75. The potency of nifedipine as an inhibitor of alpha$_1$-adrenoceptor-mediated pressor responses (Sgd 101/75 > St587 > cirazoline) was inversely related to the efficacy of the agonists (increasing proportion of spare receptors).

Differences in efficacy between agonists might therefore account for apparent selective inhibitory effects of calcium entry blockers between different agonists in the same tissue. But, more importantly, if the same agonist exhibits different efficacies in different tissues (i.e., has a different proportion of spare receptors), then it would be expected that these contractile responses would be differently affected by the same calcium entry blocker. This apparently selective effect of the calcium entry blocker(s) would, therefore, be a property of the agonist and of the receptor with which it interacts, rather than due to differences in calcium channels and their affinity for calcium entry blockers as was originally thought.

3. Calcium channel subtypes and tissue selectivity. The possibility of the existence of different types of calcium channel has already been raised (b above). The assumption often made (87) is that there are two calcium channel types, one activated by depolarization of the cell membrane (a POC) and the other activated by agonists (a ROC). This distinction has been proposed from smooth muscle studies showing that (*a*) some agonists cause a sustained contraction in K^+-depolarized preparations and (*b*) some arterial preparations may be adrenergically activated without change in membrane potential, but with concomitant increase in Ca^{2+} influx (see ref. 87 for references). Some cell types (e.g., platelets, mast cells; see section II F) seem to be devoid of voltage-dependent channels. However, mast cells are thought to possess particular calcium channels operated by IgE receptors.

Many studies have been made in smooth muscle tissues bathed in solutions containing increased concentrations of K^+ (usually 40 to 140 mM) that are known to depolarize the cell membrane (120, 535). Any differences in sensitivity of the evoked contractions under these conditions could probably be interpreted as differences between the calcium channels activated in the different tissues (neglecting the possible influence of "spare" calcium channels), the assumption being that depolarization, particularly full depolarization of the cell membrane, will fully activate a population of calcium channels (87). Some such differences have been demonstrated (22, 789, 1064, 1102, 1176) (table 2). What is less clear is the relationship between the sensitivity of these contractions to calcium channel inhibitors and the sensitivity of maximal agonist (full agonist) induced contractions in the same tissue. Van Breemen and coworkers (136, 740, 741, 1092) have shown that, in rabbit aorta and mesenteric vessels (see section II A, 2 and 5), the influxes of ^{45}Ca evoked by norepinephrine and K^+-depolarization are additive, selectively inhibited by some calcium entry blockers, and differently modulated by cyclic AMP. Moreover, Yamamoto et al. (1175) found that, in rabbit aorta, Bay K 8644 selectively opened POCs without activating ROCs. It has been argued that differences in sensitivity of ROCs and POCs to the calcium channel blockers reflect differences in calcium gating mechanisms, if not in the channels themselves. A definitive demonstration of differences between receptor-operated and potential-operated calcium channels will have to be made using electrophysiological methods. However, it has been shown in

rat aorta that, while the yohimbine isomers rauwolscine and corynanthine both inhibit K^+-induced contractions over the same concentration range (μM), only rauwolscine inhibits contractions induced by maximal concentrations of PGF_{2alpha}. This would seem to imply at least two types of calcium channel in this tissue, but unfortunately the active concentrations of the antagonists used are high, and rauwolscine may also have intracellular effects (432).

Evidence for more than one type of calcium channel activated by depolarization in smooth muscle cells was put forward by Hurwitz et al. (543). These authors found that addition of calcium to depolarized calcium-free guinea pig ileum elicited a triphasic contractile response dependent on extracellular calcium. An initial phasic contraction was followed by a rapid fall in tension and then a slowly developing contraction which gradually relaxed to a stable submaximal level of contraction. Lanthanum (6 to 20 μM) selectively inhibited the initial phasic contraction, while a higher concentration of lanthanum (0.5 mM) and verapamil (2 μM) inhibited both contractile phases to the same extent. Only the initial phasic contraction was resistant to a prolonged incubation (60 min) in calcium-free depolarizing solution. These authors proposed several calcium channels sensitive to changes in membrane potential. In other cell types (ventricular and neuronal cells), several subytpes of voltage-dependent Ca^{2+} channels have been demonstrated using "patch clamp" techniques (see section III B).

As far as receptor-operated channels are concerned, the general, and perhaps dangerous, assumption (see section III F 1 above) is that stimulation of any receptor that produces a response dependent on extracellular calcium activates a Ca^{2+} channel having similar characteristics. Recently it has been suggested that there may be subtypes of ROCs activated by serotonin and acetylcholine in bovine coronary artery (895). This suggestion rests in part on a differential antagonism of acetylcholine- and serotonin-induced contractions by diltiazem and verapamil. However, both antagonists were preincubated with the tissues for 10 min before exposure to agonist, and it is unlikely that either was at equilibrium. In rabbit aorta, bepridil, but not nifedipine or diltiazem, inhibited noradrenaline-induced contractions (by about 20%) and the associated ^{45}Ca influx (289). This difference, which seems to imply the presence of a ROC different to those described in many other tissues, is surprising.

Nevertheless, dihydropyridines do exhibit differing orders of potency as antagonists of serotonin-induced contractions in different vessels that are dependent on extracellular Ca^{2+} (22, 1064). An optimistic view, therefore, is that there are probably sufficient differences in the sensitivites of calcium channels in different vascular beds to calcium entry blocking compounds to make the search for tissue-selective compounds worthwhile and of potential great therapeutic benefit.

IV. Main Clinical Uses

Some 10 yr ago (299), the therapeutic indications for calcium antagonists were limited to cardiovascular disorders. They are now expanding outside this field to neurological, pulmonary, and urogenital disorders (44, 291, 656). A comprehensive analysis of these clinical uses is beyond the scope of this review, but the main clinical indications are shown in table 16. Some of them are briefly described below.

A. *Cardiovascular Indications*

1. Arrhythmias. Because of its potent activity on the slow Na^+-Ca^{2+} channel and its action on outward current,

TABLE 16
*Clinical applications of Ca^{2+} antagonists**

Level of proof	Clinical condition	Current preferred use†	Level of use
Good	Cardiovascular		
	Exertional angina	I, II, III	+++
	Angina at rest		
	Unstable angina	I, II, III	+++
	Prinzmetal's angina	I, II, III	+++
	Paroxysmal supraventricular tachyarrhythmias	I, III	+++
	Atrial fibrillation and flutter	I, III	++
	Hypertension	I, II, III	+++
	Neurological		
	Prophylaxis of migraine	II, IV	++
	Vertigo	IV	+++
Reasonable	Hypertrophic cardiomyopathy	I	++
	Valvular incompetence/heart failure	II, III	+
	Cerebral vasospasm secondary to subarachnoid hemorrhage	II	++
	Cardioplegia	I, II, III	+
	Raynaud's phenomenon	I, II, III, IV	++
Under examination	Protection against myocardial ischemia		
	Infarct size reduction	I, II, III	+
	Ischemic ventricular arrhythmias	I, III	+
	Primary pulmonary hypertension	II	++
	Protection against cerebral anoxia and ischemia	I, II, IV	+
	Epilepsy	IV	+
	Vertebro-basilar transient ischemic attacks	I, II, IV	++
	Leg ischemia	IV	++
	Dysmenorrhea	II	+

* This table has been established by a WHO committee.

† I, verapamil like (e.g., verapamil, gallopamil); II, nifedipine like (e.g., nifedipine, nicardipine, nimodipine, nitrendipine); III, diltiazem like (e.g., diltiazem); IV, flunarizine like (e.g., cinnarizine, flunarizine).

verapamil appears to be effective in the treatment of several forms of cardiac arrhythmias (849). It has been shown to be active in atrial fibrillation (959) and in paroxysmal supraventricular tachycardia. These actions are likely to be due to prolongation of atrioventricular nodal refractoriness by a direct action on the node proximal to the bundle of His (303).

2. *Angina pectoris.* The most spectacular therapeutic effects of calcium entry blockers have been obtained in the treatment of Prinzmetal's angina (634, 644, 869, 1129), and it is now acknowledged that coronary spasm has a prominent pathogenic role in this disease (105, 725). Furthermore, there is substantial evidence that calcium entry blockers are of value in the treatment of classic, stable, effort-induced angina (572, 655). The imbalance between myocardial oxygen supply and demand, responsible for ischemia in stable angina, can be ameliorated by augmenting the supply, reducing the demand, or both. Studies in vivo, namely by the cold pressor test, and in vitro on isolated human coronary arteries (section II A 4) have clearly demonstrated that they contract in response to various stimuli. The contraction can be attenuated by nifedipine at concentrations equivalent to those found in plasma of treated patients (428). The beneficial effect of calcium entry blockers in stable angina can also be attributed to some decrease in preload and afterload resulting from depression of venomotility and to decrease in tone of resistance vessels (380, 583, 786, 971, 1027, 1124, 1139).

3. *Hypertension.* The therapeutic effect of calcium entry blockers in the treatment of systemic hypertension has been established for diltiazem, verapamil, and nifedipine. It has been reported that the hypotensive action is observable only in hypertensive patients and that normotensive patients receiving a similar posology show no change in blood pressure. Such clinical observations have been interpreted on the basis of animal studies showing the existence of abnormalities in the properties of hypertensive vessels (620, 670). The question remains open as to whether calcium entry blockers are effective because of their vasodilating action or by reversing a primary pathogenic change. In vitro experiments show that calcium antagonists of the dihydropyridine type do restore relaxation properties in vessels from hypertensive animals. There is no doubt that further studies will help to clarify this important question and solve the problem of sustained antihypertensive therapy (412).

In spite of the decrease in blood pressure, it has been observed that nifedipine and other calcium entry blockers increase urinary volume and sodium excretion, an action that will undoubtedly contribute to their antihypertensive action (250, 546, 621, 655, 684, 842).

4. *Peripheral vascular disease.* One possible field of application of calcium antagonists is intermittent claudication. Careful studies conducted with flunarizine have shown that patients were improved, but mainly in the case of cigarette smokers. There are some encouraging reports in other peripheral vascular diseases (936).

5. *Other cardiovascular indications.* Some other indications may still be considered as being experimental. This is the case for pulmonary hypertension and hypertrophic cardiomyopathy (595, 694, 1119), while the possible antiatherosclerotic effects of calcium entry blockers, e.g., nifedipine, verapamil, nimodipine, and flunarizine (reviewed in refs. 68, 303, 504, and 857), are controversial (803, 1025). An interesting possibility is the preservation of myocardium against ischemic damage, an action that could reduce the extension of the primary necrotic area of infarcted myocardium. Encouraging, but yet preliminary, results have been reported (164, 292).

B. Neurological Indications

There are now several reports on the beneficial effect of flunarizine and nimodipine in the treatment of common and classical migraine. The pharmacological target for this action (cerebral arteries or neurones) remains controversial (354, 373, 1002).

Clinical trials are also in progress to examine the protective action of specific calcium entry blockers in ischemic brain damage. Current data in patients with aneurysmal subarachnoid hemorrhage indicate that nimodipine, a calcium entry blocker with a predilective action on the cerebral circulation, could improve the clinical situation of patients at risk (14).

Clinical reports have demonstrated a beneficial action of cinnarizine and flunarizine in the treatment of vertigo (1171) and in sleep disorders, in aged patients. Recent preliminary data suggest a possible action in some forms of epilepsia (851). Such agents, therefore, appear to be active in disseminated nervous disorders.

C. Other Indications

Variable results have been obtained with calcium entry blockers in asthmatic patients (section II B 2; ref. 44). Some calcium entry blockers are under investigation for treatment of premature labor and dysmenorrhea (see section II B 4). Dihydropyridines might be useful as antimetastatic agents (538).

V. Conclusions

A. The Pharmacotherapeutic Cascade

This brief summary of the clinical indications clearly illustrates that diltiazem, flunarizine, nifedipine, and verapamil, which may be classified as selective calcium entry blockers, do not display identical therapeutic indications (see table 16). There are indeed pharmacokinetic differences between these agents, but they are unlikely to explain such differences in therapeutic efficacy. It seems therefore worthwhile to examine the links between the effects observed at different levels of action.

The pharmacological actions of any drug may be stud-

ied at various levels as shown in table 17, which illustrates in five steps a pharmacotherapeutic cascade from the molecular to the clinical level. Before assuming the existence of a link between the various steps, some consistency should exist in the order of magnitude of the quantitative parameters estimated for the various levels of action, although this relation may be obscured by several factors (624).

An analysis of the pharmacotherapeutic cascade is possible for the action of nifedipine in angina pectoris. There is sufficient agreement between the various values obtained on rat aorta preparations to conclude that the effects observed at the tissue level [K^+-stimulated ^{45}Ca influx, IC_{50} 1.6 nM; K^+-stimulated contraction, IC_{50} 1.3 nM (402)] are related to a molecular interaction with calcium channels {[^{3}H]nitrendipine binding, K_i 4 nM (1157)}. When human preparations are considered, there is no information at the molecular level, but there is a consistency between tissue pharmacology and therapeutics, since nifedipine effects on human coronary arteries in vitro (figs. 13 and 14) are observed at concentrations close to therapeutic free drug plasma levels (7 to 20 nM) (503). Comparison of IC_{50} values of nifedipine in rat aorta and human coronary arteries shows that they are of the same order of magnitude.

The interpretation of the pharmacotherapeutic cascade is less obvious when the cardiac effects of dihydropyridines are considered, since in that case K_i values (level 1) are much lower than the relevant parameters at other levels. As discussed previously (section III, A and B), it is likely that the discrepancy is attributable to the marked voltage-dependency of the interaction of dihydropyridines with their cardiac receptor. Moreover, cardiac effects in vivo (levels 4 and 5) may be blurred by other factors, in particular activation of autonomic reflexes (section II C).

B. An Attempt at Classification of Drugs Affecting Calcium Movements (Calcium Modulators)

As proposed in table 1, drugs affecting calcium movements may be divided into inhibitors and facilitators.

TABLE 17
The pharmacotherapeutic cascade

Level of pharmacological action	Qualitative effect	Quantitative parameter
1. Molecule	Binding to Ca^{2+} channel	K_d, K_i
2. Cell	Changes in Ca^{2+} fluxes and action potentials	IC_{50}
3. Tissue (in vitro)	Cardiac negative inotropic and chronotropic effects, smooth muscle relaxation	IC_{50}, pA_2
4. Organ (in vivo)	Hemodynamic modifications (ECG, cardiac output, blood pressure)	Plasma levels
5. Clinical disorder	Antianginal and antihypertensive effects	Plasma levels

Inhibitors, also called "calcium antagonists," antagonize Ca^{2+} movements and/or functions, whereas facilitators augment Ca^{2+} movements. A useful way to subclassify calcium modulators is to take into account the subcellular localization of their site of action (407), as was done in table 1. Among calcium antagonists acting at the plasma membrane, the vast majority of drugs, designated calcium entry blockers, are thought to inhibit Ca^{2+} influx through calcium channels. As discussed above, the identification of calcium entry blockers relies on a critical comparison of the parameters of levels 1 to 3 of the pharmacotherapeutic cascade.

Selective calcium entry blockers (group I) may be defined as agents that act selectively on calcium channels operated by membrane depolarization or other stimuli. These drugs inhibit Ca^{2+} influx at concentrations that are distinctly lower than those influencing other cellular systems (e.g., adrenoceptors for verapamil, calmodulin for flunarizine). Drugs classified under subgroup I A are able to block slow calcium channels in myocardium, whereas flunarizine and cinnarizine (subgroup I B) have no perceived actions on the slow inward current in myocardium (voltage-clamp), but behave as selective calcium entry blockers in arteries.

Nonselective calcium entry blockers (group II) affect calcium channels at concentrations that are similar to, or greater than, those influencing other cellular processes. Agents belonging to subgroup II A block both Ca^{2+} and Na^+ channels. They may have interesting clinical properties, but can also present some of the side effects of local anesthetics (for instance, they may induce rhythm disturbances). Agents classified under subgroup II B form a heterogeneous group of pharmacological agents, for which the primary site of action is not the calcium channels or remains a matter of controversy [e.g., loperamide (911)].

Na^+-Ca^{2+} exchange inhibitors antagonize Ca^{2+} movements elicited by changes in Na^+ gradient. This property seems to be associated with some amiloride derivatives, but is clearly not shared by drugs of group I. The pharmacology of Na^+-Ca^{2+} exchange inhibitors is just beginning. Agents acting within the cell have been characterized mainly in studies using subcellular fractions or purified calmodulin.

Interest in the pharmacology of facilitators has increased since the discovery of the "Ca^{2+} agonist" dihydropyridines. Because some of them are partial agonists, they might find some clinical applications. As these compounds are very effective inotropic agents, it is certainly tempting to search for derivatives that would be more selective for myocardium.

Our classification of calcium entry blockers presents some differences with those proposed by other investigators. Fleckenstein (303) has classified "specific calcium antagonists" into two groups, A and B, which correspond to subgroups I A and II A in table 1. He did not include diphenylpiperazines in his classification, because these

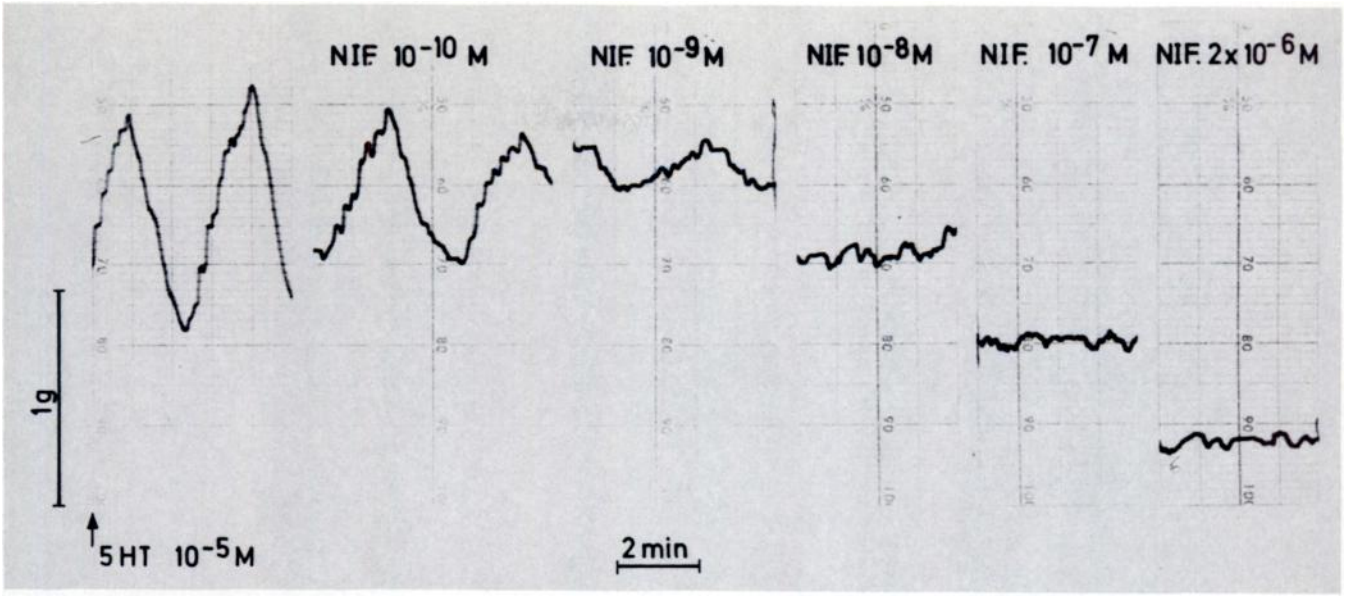

FIG. 14. Effect of nifedipine at increasing concentrations on the increase in basal tone and on the rhythmic contractile activity induced by serotonin in an isolated segment of a 9-yr-old human coronary artery. *NIF*, nifedipine; *5 HT*, serotonin. Reproduced from Godfraind et al. (413).

drugs could not be satisfactorily characterized in electrophysiological experiments on myocardial tissue. Another classification into three groups (dihydropyridines, verapamil and diltiazem, diphenylpiperazines and related drugs) has been advocated by Spedding (1008, 1013, 1016) after a comparison of the actions of calcium entry blockers on isolated *taenia coli* preparations. The main criteria taken into consideration were (*a*) the effect of changing surface charge with salicylate (1009) on the inhibitory action on K^+-depolarized preparations and (*b*) the functional interactions between calcium entry blockers and Bay K 8644 (1013). Classifications and models based on binding studies have been discussed in section III A.

Another useful approach (408) is to classify selective calcium entry blockers on the basis of the following functional criteria (fig. 15).

1. cardiac effects resulting mainly from inhibition of slow inward current: antiarrhythmic action; slowing of conduction (rhythm); and negative inotropic effects (inotropy)
2. vascular effects: inhibition of myogenic contraction of rat portal vein (myogenic) and of contractions of isolated arteries (arteries)
3. tissue protection: against myocardial necrosis due to intracellular calcium overload resulting from anoxia, ischemia, or isoproterenol intoxication (protection, cardiac); against ischemic and anoxic alterations of brain tissue (protection, brain)
4. hemorrhagic effects: restoration of red blood cell deformability altered by calcium-induced stiffness (RBC deformability).

On the basis of their effects in these different bioassays, the existing drugs can be subdivided into three categories that are similar to those of Spedding (1008).

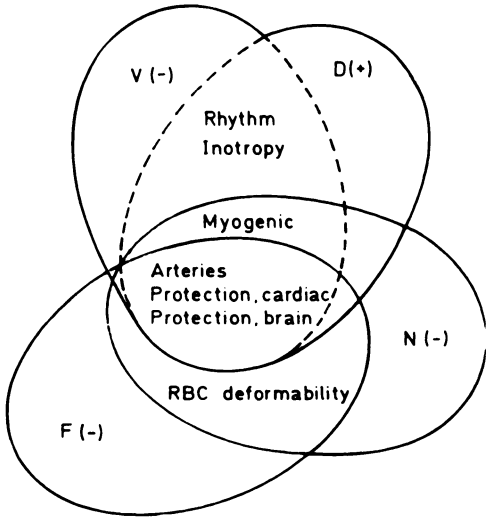

FIG. 15. Differentiation of selective calcium entry blockers on the basis of functional criteria and of binding data. *V*, verapamil like; *D*, diltiazem like; *N*, nifedipine like; *F*, flunarizine like. *Plus* and *minus signs* refer to stimulation and inhibition of [^{3}H]dihydropyridine binding, respectively. *Rhythm* and *inotropy* refer to cardiac effects as observed in vivo, which are more pronounced with verapamil and diltiazem than with nifedipine (section II C). For further explanations, see text.

Taking into account the action of the drugs at the molecular level, specifically their interaction with [^{3}H] dihydropyridine binding sites, verapamil-like drugs may be differentiated from diltiazem (see section III A). The four subclasses obtained in this manner (fig. 15) correspond to the four chemical classes of selective calcium entry blockers listed in table 1. The different clinical indications for diltiazem, verapamil, nifedipine, and flunarizine (table 16) are consistent with this classification, which emphasizes the importance of tissue selectivity in

TABLE 18
Glossary of generic or chemical names

Compound designation	Generic or chemical name
A 23187	Calimycin
AQ-A-39	Falipamil
Bay e 6927	2,6-Dimethyl-3-isopropyl-5-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate
Bay K 8644	Methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate
B-HT 920	6-Allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo(4,5-*d*)azepin dihydrochloride
B-HT 933	Azepexol hydrochloride
CGP 28392	4-(2-Difluormethoxy)phenyl-1,4,5,7-tetrahydro-2-methyl-5-oxofuro(3,4-*b*)pyridine-3-carboxylic acid ethyl ester
D 600	Gallopamil
D 888	Desmethoxyverapamil
D 890	N-Methyl-gallopamil
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
FR 34235	5-Isopropyl-3-methyl-2-cyano-6-methyl-4-(3-nitrophenyl)-1,4-dihydro-3,5-pyridinedicarboxylate
FR 7534	1,4-Dihydro-2-hydroxymethyl-4-(*m*-nitrophenyl)-6-methyl-3,5-pyridine dicarboxylic acid diethyl ester
Hoe 263	N-[2-(Benzhydryloxy)ethyl]-N-methyl[1-methyl-2-(3-methoxyphenyl)ethyl] amine
KB 944	Fostedil
MDL 12330A	N-(*cis*-2-Phenylcyclopentyl)azacyclotridecan-2-imine hydrochloride
PK 11195	1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolincarboxamide
PN 200-110	4-(2,1,3-Benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylic acid-methyl-1-methylethyl ester
PY 108-068	Dazodipine
Quin-2	2-Methyl-6-methoxy-8-nitroquinoline
(*R*)202-791 (*S*)202-791	Isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate
R-24571	Calmidazolium
RS 21361	2-(1-Ethyl-2-imidazolyl-methyl)-1,4-benzodioxan
Sgd 101/75	Indanidine
SKF 525A	Proadifen
St 587	2-(2-Chloro-5-trifluoromethylphenylimino)imidazoline
TMB-8	2-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate

TABLE 18

Compound designation	Generic and/or chemical name
UK 14304	2-(8-Bromoquinoxalyl-7-imino)imidazoline tartrate
W-7	N-(6-Aminohexyl)-5-chloro-1-naphthalene sulphonamide
WB 4101	2-[(2′,6′-Dimethoxy)phenoxyethylamino]methylbenzodioxane
YC-170	2-(2-Pyridil)ethyl-4-(O-chlorophenyl)-2,6-dimethyl-5-phenylcarbamoyl-1,4-dihydropyridine-3-carboxylate

the pharmacological and therapeutic actions of calcium entry blockers.

C. Concluding Remarks

The field of drugs acting on calcium movements and on calcium function is growing so quickly that we are convinced that this review has not covered all aspects of this research. A large body of information can be found in the reviews and monographs dedicated to more specialized topics (74, 303, 326, 405, 409, 414, 439, 476, 503, 527, 635, 811, 847, 976, 978, 1026, 1146). Further work is still required at all levels of the pharmacotherapeutic cascade in order to confirm that the experimental classification proposed in table 1 and fig. 15 has a prospective value in therapeutics. For instance, controlled clinical studies comparing the action of a variety of calcium entry blockers in angina would be useful in order to validate in patients the very marked differences in the action of nifedipine and diltiazem on human coronary arteries in vitro. Nifedipine completely relaxes coronary arteries contracted by PGF_{2alpha} (404, 428), whereas diltiazem relaxes this contraction by only 50% (617) (see section II A 4).

When attempting to relate the therapeutic effects of drugs to their pharmacological properties, it is important to remember that the tissue conditions prevailing in pathological states may profoundly affect the drug activity. In ischemic or anoxic conditions, a drop in the activity of the sodium-potassium pump and an activation of K^+ efflux may be responsible for an increase of extracellular K^+, which will in turn modify the membrane potential and the function of calcium channels. As already pointed out by one of us (401), we are facing a new trend in pharmacology, having drugs that show a more pronounced effect in disease and in pathophysiological models than in physiological conditions. For instance, by contrast with verapamil, flunarizine has no observable effect on the slow calcium channels of myocardial tissue, but like verapamil, it is a very powerful protecting agent against myocardial damage evoked in vivo by large doses of isoproterenol, which is attributed to intracellular calcium overload. The difficulty of demonstrating marked pharmacological effects in nervous tissue, where calcium entry blockers have been shown to have high affinity binding sites and where dihydropyridines antagonize effects of calcium agonists (751), could be due to the fact that adequate experimental conditions of activation have not generally been achieved. There is therefore a need for appropriate pathophysiological models, the predictive value of which, however, may be affected by species differences.

In this review, we have emphasized the importance of tissue selectivity in the therapeutic activity of calcium entry blockers. It remains to be determined to what extent this selectivity is due to differences in the local (tissue) regulation of calcium channels or to an uneven tissue distribution of calcium channels sensitive and resistant to calcium entry blockers. Hopefully, progress in the study of calcium channels at the molecular level will help towards a better understanding of this tissue selectivity, which should stimulate a search for novel agents with greater therapeutic specificity.

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