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Perspective

New Developments in Ca^{2+} Channel Antagonists

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Introduction

Despite the 100 years that have elapsed since the discovery by Sidney Ringer of the vital role for Ca^{2+} in the maintenance of cardiac contractility, the messenger function of Ca^{2+} has been appreciated only comparatively recently (reviewed in ref 1–3). This messenger function for Ca^{2+} (Figure 1), a consequence of a critical cellular decision probably made early in the course of evolution,[4] is made possible by three key features of cellular Ca^{2+} regulation: (1) In the resting state the intracellular concentration of ionized (free) Ca^{2+} is low ($\leq 10^{-7}$ M), but it increases during excitation to between 10^{-7} and 10^{-5} M. (2) There exist within the cell specific Ca^{2+}-binding proteins with dissociation constants for Ca^{2+} of between 10^{-7} and 10^{-5} M and which serve as intracellular Ca^{2+} receptors. (3) Within the plasma membrane and intracellular organelles, Ca^{2+}-specific entry, exit, and sequestration processes exist. These processes function both to generate the elevated levels of Ca^{2+} during excitation and to restore and maintain the low intracellular Ca^{2+} levels of the resting state.

A schematic representation of cellular Ca^{2+} regulation is shown in Figure 2. Cellular Ca^{2+} is stored in intracellular organelles, including mitochondria (MI) and sarcoplasmic reticulum (SR), by energy-dependent transport processes.[5-7] Ca^{2+} release, notably from sarcoplasmic reticulum and functionally related structures, plays an important role in stimuli that directly or indirectly mobilize intracellular Ca^{2+}. It is probable that the plasma membrane, at its cytosolic interface, also plays an important role in Ca^{2+} storage and release processes. Although both mitochondria and sarcoplasmic reticulum have significant storage capacities for Ca^{2+}, the cell must, in order to avoid the deleterious consequences of Ca^{2+} overload,[8] ultimately remove Ca^{2+} to the extracellular environment. At least two Ca^{2+} mechanisms are involved, a plasmalemmal Ca^{2+}-ATPase and a Na^+–Ca^{2+} exchange process.[9-14] The latter derives its cation countertransporting ability from Na^+,K^+-ATPase and may, according to the ratios of intracellular and extracellular Na^+, serve to remove Ca^{2+} from or introduce Ca^{2+} to the cell. Within the cell, the targets for Ca^{2+} are an homologous group of Ca^{2+}-binding proteins, including parvalbumins, troponin C, and calmodulin, that serve to confer Ca^{2+} sensitivity to mechanical, secretory, and metabolic events.[2,3,15] Of particular importance is calmodulin, since it is highly conserved in structure, has a wide-spread phylogenetic distribution, and has multiple roles in Ca^{2+}-dependent cellular regulation (Table I).[1,15-20]

Table I. Calmodulin-Dependent Events

Cellular Events	
motility	contraction of smooth muscle
axonal transport	hormone and neurotransmitter release
phospholipid breakdown	prostaglandin synthesis
cell proliferation	cell architecture
Ca^{2+} transport	
Enzyme Activation	
cyclic nucleotide phosphodiesterase	myosin light chain kinase
adenylate cyclase	phospholipase A_2
phosphorylase *b* kinase	glycogen synthase kinase
NAD^+-kinase	

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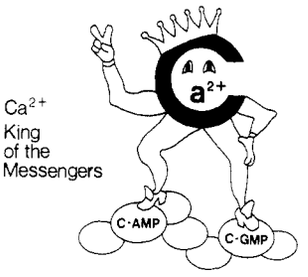

Figure 1. Ca^{2+}—king of the messengers (with apologies to cyclic AMP and cyclic GMP).

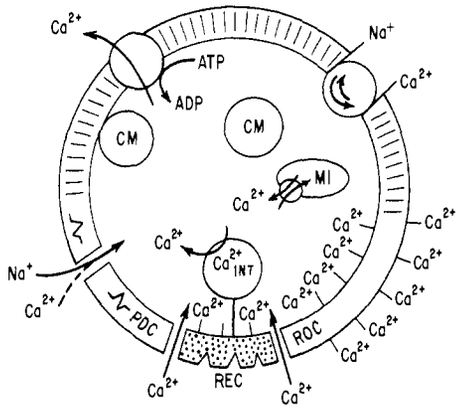

Figure 2. Schematic representation of cellular Ca^{2+} regulation. Ca^{2+} storage within the cell is shown in mitochondria (MI) and other intracellular loci (Ca^{2+}_{int}), including sarcoplasmic reticulum and the internal plasma membrane surface. Ca^{2+} entry, as discussed in the text, can occur through receptor-operated and potential-dependent channels, as well as through the Na^+ channel. Intracellular Ca^{2+} levels are regulated through the operation of membrane pumps, including Ca^{2+}-ATPase and a Na^+/Ca^{2+} countertransport. The functions of intracellular Ca^{2+} are mediated through Ca^{2+} binding proteins, notably calmodulin (CM), shown in cytosolic and membrane-associated states. Reproduced from ref 56.

To complement the several storage and efflux processes for Ca^{2+} there exist several influx pathways. Although Ca^{2+} can enter the cell through a "leak" pathway (unstimulated) and as a minor contributor to the fast inward Na^+ current,[21] it has been proposed that the two major types of Ca^{2+} entry pathways are those that have been designated potential-dependent (PDC) and receptor-operated (ROC) channels.[22,23] Potential-dependent channels have been defined as those activated by membrane depolarization (electrical or elevated K^+), while receptor-operated channels are those associated with membrane receptors and are activated by specific agonist–receptor interaction. It is not known if the channels themselves are different structures or if the association of ligand receptors with PDC changes their voltage dependence and sensitivity to channel antagonists and makes them ROC. In principle, Ca^{2+} mobilization during cellular excitation may be initiated from both extracellular and intracellular sources (Figure 3), the relative extent of which will depend on several factors, including the tissue, stimulant, species, the environment of the Ca^{2+} channels, and the effect of other Ca^{2+} regulating mechanisms.

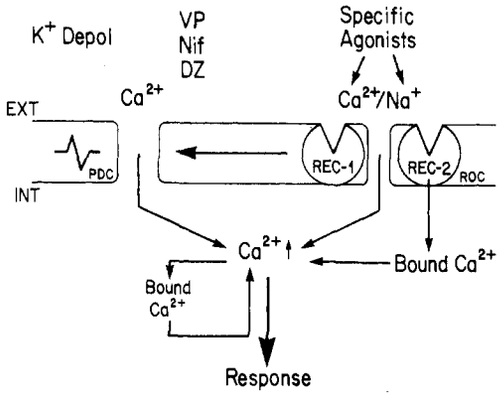

Figure 3. Ca^{2+} mobilization in response to plasma membrane signals. Two types of Ca^{2+} channels are shown, receptor-operated (ROC) and potential-dependent channels (PDC). Specific agonist–receptor interactions (REC-1, REC-2) can mobilize Ca^{2+} through ROC or from intracellular sources or may depolarize the membrane and activate the PDC. K^+-depolarizing stimuli activate PDC only. Ca^{2+} mobilization may also include regenerative (Ca^{2+} induced) Ca^{2+} release to amplify the signal for producing the response. VP, Nif, and DZ are verapamil, nifedipine, and diltiazem, respectively. Their site of action, which is probably in the Ca^{2+} channel, is not represented in the figure.

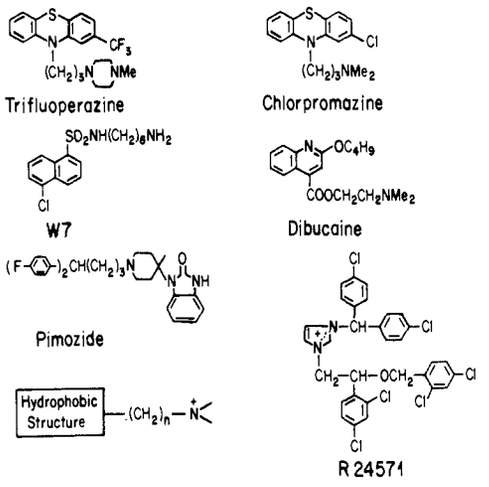

Figure 4. Structure of calmodulin antagonists, including a generalized structure.

These processes of Ca^{2+} regulation at the cellular level are paralleled by Ca^{2+} regulation at the organismic level, where body Ca^{2+}, total and plasma, is regulated by a triumvirate of agents, vitamin D, calcitonin, and parathyroid hormone, serving to regulate Ca^{2+} entry, Ca^{2+} storage, and Ca^{2+} excretion.[24]

Ca^{2+} Antagonists

The ubiquitous role of Ca^{2+} in cell regulation and the diversity of processes controlling cellular Ca^{2+} concentration indicate the importance of identification of the sources and routes of Ca^{2+} mobilization. One approach is through the use of agents that may selectively antagonize the pathways of Ca^{2+} utilization.

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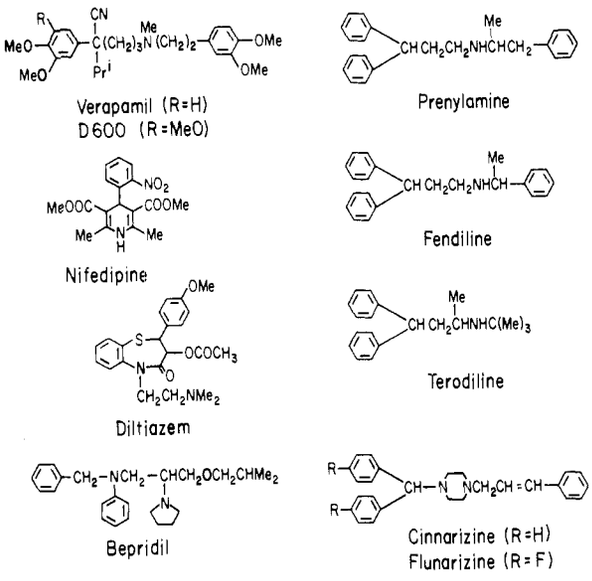

Figure 5. Structure of Ca^{2+} channel antagonists.

There is an abundance of structures possessing to some degree the ability to inhibit Ca^{2+}-dependent processes.[25,26] For most of these structures, however, their ability to antagonize Ca^{2+}-mediated processes is probably indirect and, in any event, is clearly secondary to other and better defined pharmacological activities. However, considerable attention has been paid in recent years to two major groups of compounds—the calmodulin antagonists (Figure 4) and the Ca^{2+} channel antagonists (Figure 5).

Calmodulin Antagonists. Both groups of compounds are characterized by significant heterogeneity of chemical structure, which may suggest multiple sites and mechanisms of action. However, for the compounds depicted in Figure 4, it is apparent that their ability to interact with calmodulin is dominated largely by hydrophobic interactions,[27-30] consistent with their interaction at a nonpolar site on calmodulin exposed during the prerequisite step of Ca^{2+} binding.[31] Neither binding to calmodulin nor inhibition of calmodulin-dependent phosphodiesterase by the isomers of butaclamol, thiothixene, or flupenthixol exhibits stereoselectivity,[27,32,33] observations consistent with a relatively nonspecific mode of interaction with calmodulin and in marked contrast to the stereoselectivity of these same compounds, exhibited at much lower concentrations, in inhibiting dopamine receptor binding and adenylate cyclase activation.[29,30,32,33] Nonetheless, hydrophobicity is not the sole determinant of calmodulin antagonism, and Weiss and his colleagues[29] suggest a general structure for calmodulin antagonists as shown in Figure 4.

Table II. Therapeutic Indications for Ca^{2+} Channel Antagonists

Current Uses
angina: vasospastic, unstable at rest, and chronic stable
supraventricular tachycardia
ventricular tachyarrhythmia
atrial flutter and fibrillation
hypertension
Possible Future Uses
cerebral insufficiency and vasospasm
pulmonary hypertension
asthma
premature labor
primary dysmenorrhea, myometrial hyperactivity
myocardial ischemia and failure
cardiac preservation
intestinal spasm
peripheral vascular disease
esophageal motor disorders, achlasia

The Ca^{2+} channel antagonists (Figure 5) resemble the calmodulin antagonists in that they are also a diverse group of molecular structures. Unlike the calmodulin antagonists, however, the Ca^{2+} channel antagonists are highly potent, exhibit Ca^{2+} channel antagonism as their principal pharmacological property, and possess defined structure–activity relationships, including stereoselectivity. The remainder of this review will focus on this group of Ca^{2+} antagonists.

Ca^{2+} Channel Antagonists. The drugs currently available in North America, verapamil, nifedipine, diltiazem, and lidoflazine, have a number of therapeutic indications (Table II).[34-36] These drugs are the first antianginal agents introduced to the United States within the last decade that have the potential of becoming drugs of choice for most patients with angina. The description of these agents, variously referred to as Ca^{2+} antagonists, Ca^{2+} channel antagonists, slow channel blockers, or Ca^{2+} entry blockers, owes much to the original investigations of Fleckenstein who first observed that verapamil and prenylamine mimic the cardiac effects of Ca^{2+} withdrawal.[37] Subsequent studies showed that these and a number of other agents, including nifedipine, fendiline, and perhexiline, were cardiodepressant and coronary vasodilator drugs acting in an apparently competitive fashion against Ca^{2+} and served to introduce the principle of specific Ca^{2+} antagonism to therapeutics.[38-40] Since the original studies with verapamil and prenylamine, a large number of additional structures have joined this class of Ca^{2+} antagonists (Figure 5), and it is clear that this group of compounds is neither structurally nor pharmacologically homogeneous.[34,40-43] Thus, Fleckenstein[40] (see also ref 41 and 43) has divided compounds into group A (verapamil, D600,

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Table III. Antagonist Activities in Smooth Muscles[a]

system		antagonist	ID_{50}, M
guinea pig ileum	ACh	nifedipine	5×10^{-9}
	K^+		3×10^{-9}
canine coronary	NE	D600	5×10^{-7}
	K^+		2×10^{-7}
canine trachea	ACh	verapamil	$>10^{-4}$
	K^+		$<<10^{-4}$
rabbit aorta	NE	verapamil	1×10^{-4}
	K^+		3×10^{-8}
rabbit basilar	5-HT	nimodipine	7×10^{-10}
	K^+		2×10^{-10}
rabbit saphenous	5-HT	nimodipine	$>10^{-5}$
	K^+		3×10^{-10}

[a] References 64-68.

diltiazem, and nifedipine and related 1,4-dihydropyridines) and group B (prenylamine, fendiline, terodiline, and perhexiline), based on the potent and selective effect of the group A compounds on the Ca^{2+} component of the cardiac action potential and the less selective effect (concomitant inhibition of Na^+ current) of the group B compounds. A further division can be made on the basis of the cardioselectivity and vascular selectivity of these agents. Verapamil, D600, and diltiazem are approximately equiactive in cardiac and vascular smooth muscle, whereas nifedipine (and other 1,4-dihydropyridines), flunarizine, and cinnarizine are clearly more selective for vascular smooth muscle.[38-41,43-46] These differences relate to their relative therapeutic utilities and are consistent with significant differences in sites or mechanisms of action.

From these preliminary considerations a number of questions may be posed concerning the Ca^{2+} channel antagonists: (1) Specific actions (stimulus selectivity, structure–activity relationships, stereoselectivity)? (2) Relationship to Ca^{2+} (inhibition of Ca^{2+} currents, competitive to Ca^{2+}, inhibition of Ca^{2+} uptake)? (3) Selectivity (stimuli, tissues)? (4) Sites and mechanisms of action (Ca^{2+} channels, other membrane sites, multiple mechanisms, sidedness, state-dependent block)? Recent reviews have summarized much basic and clinical data on the Ca^{2+} channel antagonists.[25,26,35,36,39,46-56]

(1) Specific Actions. Specific actions of the Ca^{2+} channel antagonists at a defined subgroup of Ca^{2+} mobilization processes are strongly indicated by the stimulus selectivity exhibited by these agents. In smooth muscle, depolarization-induced responses are usually very sensitive

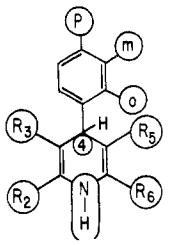

Figure 6. Structure–activity requirements in the 1,4-dihydropyridine series. For further discussion see text.

to these drugs, but agonist-induced responses show great variation in sensitivity (Table III); extensive compilations of similar data have been provided by Flaim[54] and Cauvin et al.[50]

The generally high sensitivity of K^+-induced responses and the variable sensitivity of agonist-induced responses may reflect the varying extent of agonist-induced Ca^{2+} mobilization through PDC relative to ROC and intracellular sources. Implicit in this proposal is that PDC and ROC are relatively sensitive and insensitive, respectively, to the Ca^{2+} channel antagonists.[25,26,55,56] Alternatively, the ROC may display a range of sensitivities to the Ca^{2+} channel antagonists according to the receptor and tissue in question.[23,50,57] These possibilities remain to be critically distinguished, but some agonist-induced contractions are more sensitive to the Ca^{2+} antagonists than are K^+-induced contractions.[58-60] Furthermore, in rabbit aorta the effectiveness of nisoldipine and diltiazem as inhibitors of norepinephrine (NE) induced contraction and $^{45}Ca^{2+}$ uptake decreased dramatically with increasing NE concentration, and van Breemen[50,57] has suggested the existence of multiple activated ROC states characterized by varying susceptibility to antagonist blockade. It has been previously proposed[52,61] that the sensitivity of Ca^{2+} channels to blockers is dependent on the many membrane and intracellar factors that are known to regulate Ca^{2+} channel function.[62,63] According to this view, the PDC of smooth muscle that are highly sensitive and the ROC channels that are less sensitive to Ca^{2+} channel antagonists may represent two commonly occurring Ca^{2+} channel states in a continuum of potential states for a single basic type of smooth-muscle Ca^{2+} channel.

A specific site of action, rather than, for example, some nonspecific membrane perturbation, is also indicated by the existence of strict structure–activity relationships, including stereoselectivity.[25,26,47,55,56,69] In a comparison of

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the negative inotropic effects of 15 agents, including both Ca^{2+} antagonists and other compounds (diphenylhydantoin, diazoxide, flurazepam, etc.), a positive association of activity and increasing lipophilicity was observed for the latter compounds, consistent with a membrane perturbant action.[69] Structure–activity data for 1,4-dihydropyridines are available for several systems,[42,70–73] including in vivo blood pressure,[69,71] isolated cardiac (papillary) muscle,[70] and intestinal smooth muscle.[25,64,74] The general structural requirements for activity are summarized in Figure 6 and indicate the following: (a) The 1,4-dihydropyridine ring is essential. Oxidation to the pyridine abolishes activity. (b) The NH group of the 1,4-dihydropyridine ring must be unsubstituted for optimum activity. (c) The 2,6-substituents of the 1,4-dihydropyridine ring should be lower alkyl, although one NH_2 group is tolerated. (d) Ester substituents in the 3- and 5-positions of the 1,4-dihydropyridine ring are optimum. Removal or replacement by COMe or CN greatly reduces activity. (e) Ester substituents larger than COOMe generally maintain or even increase activity, suggesting a region of bulk tolerance in the site of 1,4-dihydropyridine interaction. (f) An aryl substituent, preferably a substituted phenyl group, appears optimum for the 4-position of the 1,4-dihydropyridine. The position of the substituent in the phenyl ring is critical: para substitution invariably decreases activity, whereas ortho or meta substitutions generally increase activity according to their electronic and steric effects. (g) When the ester substituents at C_3 and C_5 of the dihydropyridine ring are different, the C_4 position becomes chiral, and stereoselectivity of antagonism is observed.[64,72,75–79]

Only limited quantitative structure-activity relationships (QSAR) have been delineated for the 1,4-dihydropyridines.[42,55,56,70] Thus, for a small series of 2,6-dimethyl-3,5-dicarbomethoxy-4-substituted-phenyl-1,4-dihydropyridines, the effects of the phenyl substituent (ortho or meta) on negative inotropic potency correlated with the Verloop steric parameter B_1,[80] increasing with increasing B_1 values. Loev et al.[69] had earlier suggested that the effect of *o*-phenyl substituents might be to ensure a perpendicular orientation of the phenyl ring to the 1,4-dihydropyridine ring. A role for steric influences is also suggested from the solid-state structures of a small number of 1,4-dihydropyridines,[81] where, despite the well-known difficulties of extrapolation from solid-state to receptor-bound conformations, a correlation has been observed between the extent of 1,4-dihydropyridine ring pucker and pharmacological activity. Substituents (ortho or meta) in the phenyl ring influence the 1,4-dihydropyridine ring conformation, activity increasing with increasing ring planarity: as noted previously para substituents in the phenyl ring did not obey this relationship, suggesting that the highly detrimental effect of these substituents arises from hindrance to the actual receptor interaction.

Table IV. Ca^{2+} Antagonist Actions in Cardiac Tissue[a]

preparation	activity	antagonist	ID_{50}, M
guinea pig papillary	contraction	verapamil	5×10^{-7}
guinea pig atria	contraction	D600	$<10^{-7}$
		nifedipine	10^{-7}
cat papillary	Ca^{2+} current	D600	2×10^{-6}
		nifedipine	6×10^{-7}
cat ventricular trabeculae	Ca^{2+} current	nifedipine	6×10^{-7}
		verapamil	$<10^{-6}$
guinea pig single ventricular cells	Ca^{2+} current	nitrendipine	2×10^{-7}

[a] References 26, 83, 88, and 89.

Table V. Inhibition of $^{45}Ca^{2+}$ Uptake and Mechanical Response in Smooth Muscles[a]

		ID_{50}, M	
system	antagonist	contraction	$^{45}Ca^{2+}$ uptake
rabbit pulmonary artery, K^+	verapamil	4.0×10^{-7}	3.0×10^{-7}
rabbit aorta, K^+	D600	2.0×10^{-7}	1.5×10^{-7}
	verapamil	1.7×10^{-7}	6.8×10^{-9}
	nicardipine	1.9×10^{-9}	1.0×10^{-9}
	diltiazem	5.0×10^{-7}	3.0×10^{-7}
rat aorta, K^+	flunarizine	2.2×10^{-7}	1.8×10^{-7}

[a] References 23, 50, and 90–92.

QSAR data for other classes of Ca^{2+} channel antagonists is quite limited. For a series of verapamil analogues, substituted in the phenyl ring adjacent to the asymmetric carbon, the best correlation obtained was with the *F* substituent constant, indicating the importance of the electron-withdrawing ability of the substituent.[82] Further evidence for specificity of actions is provided by the stereoselectivity of both verapamil and D 600, (–) > (+), observed in both cardiac- and smooth-muscle preparations.[25,83–87] It is clearly important for both the 1,4-dihydropyridine and the verapamil series that larger groups of analogues be systematically analyzed to test the significance of the limited correlations thus far achieved.

(2) Relationship to Ca^{2+}. Vital to the original definition of Ca^{2+} antagonists were the observations that their cardiodepressant and smooth-muscle relaxant actions mimicked Ca^{2+} withdrawal and that these actions were overcome by elevation of the extracellular Ca^{2+} concentration.

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Very direct evidence supports the conclusion that this group of antagonists does block a specific sarcolemmal Ca^{2+} entry process. Myocardial Ca^{2+} currents are blocked at concentrations close to those necessary to produce electromechanical uncoupling (Table IV). Similarly, activated (but not resting) Ca^{2+} uptake is blocked by the Ca^{2+} antagonists,[23,50,55,56,64,88] and where complete dose–response relationships are available, there is good agreement between inhibition of mechanical response and inhibition of Ca^{2+} uptake (Table V). However, exceptions have been noted[93-95] in which a dissociation of inhibitory effects on mechanical response and Ca^{2+} uptake occur. The origins of these discrepancies, limited in number, are not understood, and it is clear that the majority of studies do find a close link between the inhibitory effects of Ca^{2+} antagonists on mechanical responses and Ca^{2+} uptake. These results argue against mechanisms of action other than those that result in decreased ^{45}Ca uptake.

The relationship between Ca^{2+} and the Ca^{2+} antagonists has often been described as competitive. Thus, in depolarized smooth and in cardiac muscle, dose–response curves to Ca^{2+} are shifted to the right and a series of pA_2 values have been reported for Ca^{2+} antagonists.[43,56,87,96-99] However, it is not clear that a true competitive relationship has always been established: the concentration range over which $[Ca^{2+}]_{ext}$ can be varied makes accurate determination of the slopes of Schild (dose ratio) plots difficult, and many pA_2 values have been determined in single point assays. Where slope values are available, they are often significantly different from unity.[42,43,87,96] Furthermore, there exist discrepancies; for example, in depolarized rabbit aorta the effects of diltiazem on Ca^{2+}-induced responses become progressively less reversible by Ca^{2+} with increasing diltiazem concentration.[97,100] The reversible inhibition of Ca^{2+} and Ba^{2+} current by verapamil, diltiazem, and nitrendipine is also not consistent with simple competition at the cation binding site at which inorganic cations block the Ca^{2+} channel,[88] this binding site being the Ca^{2+} coordination site for Ca^{2+} movement through the channel.[62] The results of Lee and Tsien[88] suggest that there are at least two different sites of Ca^{2+} interaction as it permeates the Ca^{2+} channel, an outer site, where competition between Ca^{2+} and Cd^{2+} occurs, and an inner site, where competition with organic blockers occurs. Their results with cardiac cells also indicate that the increased Ca^{2+} concentration gradient is not the reason for the increased current when extracellular Ca^{2+} is elevated.

Major questions thus remain to be resolved concerning the interaction between Ca^{2+} and the Ca^{2+} antagonists. It is possible that there is competition for a common binding site that is modulated by the state of the Ca^{2+} channel or that the apparent competitive behavior may have other origins, including allosteric interactions between the cation and drug binding sites, recruitment of additional Ca^{2+} channels by elevated Ca^{2+}, or activation of "spare" Ca^{2+} channels. It is noteworthy that Spedding[98] reported that the calmodulin antagonists pimozide and trifluoperazine behaved similarly to the Ca^{2+} channel antagonists, producing a rightward shift of the Ca^{2+} dose–response curve in depolarized intestinal smooth muscle. Thus, the shift seen with Ca^{2+}-channel antagonists does not itself necessarily indicate a competitive blockade of Ca^{2+} entry.

(3) Selectivity. The question of selectivity of Ca^{2+} channel antagonist action is of fundamental importance. In principle, selectivity may be considered at two levels—stimulant and tissue. Examples of stimulant selectivity have already been provided (Table III) and probably reflect, at least in part, the relative extent to which Ca^{2+} is mobilized by the stimulant through potential-dependent channels, receptor-operated channels, and intracellular sources. Thus, the nifedipine analogue nimodipine is very effective against 5-hydroxytryptamine (5-HT) responses in rabbit basilar artery but is ineffective against 5-HT responses in rabbit saphenous artery.[68] Similarly, responses in rat vascular tissue mediated through postsynaptic α_2 receptors are sensitive to the Ca^{2+} channel antagonists, whereas responses mediated through α_1 receptors are insensitive.[101-104] The extent to which stimulant selectivity is observed may also be dependent upon the stimulant concentration. Thus, in canine tracheal smooth muscle the responses to low and high concentrations of acetylcholine are sensitive and insensitive, respectively, to the Ca^{2+} antagonists,[66] and similar observations have been made for norepinephrine responses in rabbit aortic smooth muscle.[100]

However, it is also possible that differences in Ca^{2+} antagonist sensitivity are exerted at the tissue level. Such differences may have a variety of origins: pharmacokinetic differences; physiological reflexes; relative agonist use of PDC; Ca^{2+} channel differences, (a) binding and (b) kinetics; frequency and voltage dependence of interaction; pathological state of tissue.

Of particular importance is the question of whether differences in Ca^{2+} channel organization or kinetics are recognized by the existing Ca^{2+} channel antagonists and are translated into selectivity of action. Several lines of evidence indicate that such differences may exist.

Significant differences in the relative cardiac depressant/smooth-muscle relaxant activities between the three major agents verapamil, diltiazem and nifedipine are well recognized.[25,34,35,39,41,50,51] Verapamil and diltiazem are approximately equiactive in cardiac and smooth muscle, whereas nifedipine is significantly more active in smooth muscle (vascular and nonvascular). These differences are observed both in vivo and in vitro and are reflected in the

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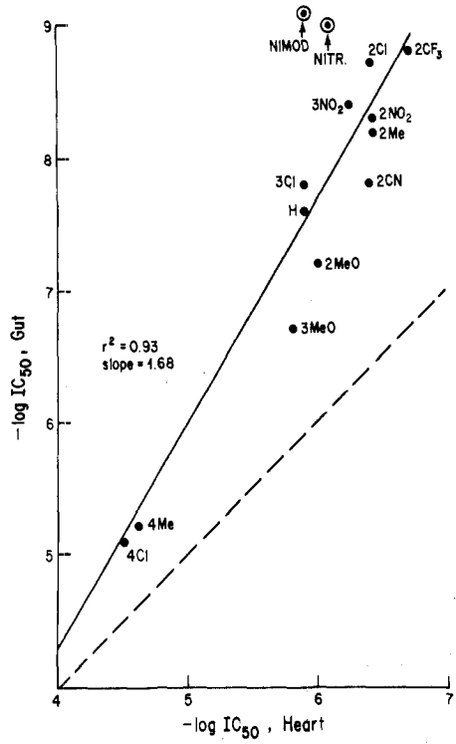

Figure 7. Correlation of pharmacological activities of a series of 2,6-dimethyl-3,5-dicarbomethoxy-4-substituted-phenyl-1,4-dihydropyridines in gut (acetylcholine contractions in guinea pig ileal longitudinal muscle) and cardiac (negative inotropic activity in cat papillary muscle) preparations. The solid line is that of best fit, and the dashed line is for 1:1 equivalence. The heart data are from ref 70. NIMOD and NITR are nimodipine and nitrendipine, respectively.

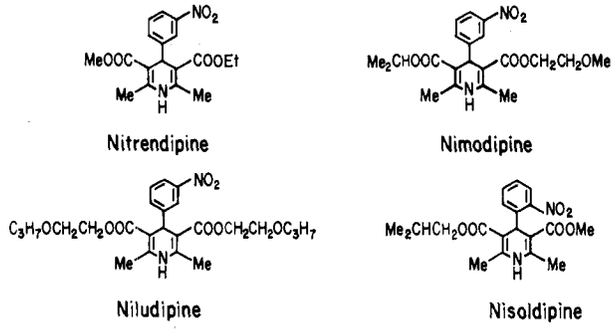

Figure 8. Some selectively acting 1,4-dihydropyridines.

clinical indications for these agents.

The higher activity of the dihydropyridine series in smooth muscle is revealed very clearly in the data of Figure 7 comparing the inhibitory activities of a series of phenyl-substituted nifedipine analogues in cat papillary and guinea pig intestinal muscle. Despite this major difference in sensitivity, the structural requirements for activity are clearly very similar in both tissues. The significant deviation of nimodipine (and to a lesser extent nitrendipine) from this correlation is of particular interest, since these are ester-substituted analogues of nifedipine (Figure 8). This deviation parallels a previous report that niludipine (Figure 8) shows a greatly enhanced smooth-muscle selectivity relative to nifedipine,[43] and nisoldipine (Figure 8) is also more potent than nifedipine as a vasodilator but has the same cardiac activity.[105] Substitution at the ester position in the 1,4-dihydropyridine series (Figure 6) may thus be a probe for differences in binding site organization or channel function in cardiac and smooth muscle. Whether such differences also exist between individual smooth muscles or vascular beds is uncertain. However, it has been reported that nimodipine exhibits selectivity for the cerebral vasculature.[68,106] Further examination of the basis of tissue selectivity is clearly an area worthy of much serious investigation.

Although major emphasis in the actions of Ca^{2+} channel antagonists has been directed toward excitation–contraction coupling, data are available for a number of stimulus–secretion coupling systems. Quite generally, these systems are less sensitive to the Ca^{2+} antagonists than are smooth and cardiac muscle contraction.[25,26,55] Thus, in many neuronal systems, calcium currents and depolarization-induced Ca^{2+} uptake[107-112] are affected only at very high concentrations of antagonist, where it is not likely that specific channel blockade is occurring. However, Ca^{2+} current in *Helix* neuron is relatively sensitive to nitrendipine,[113] and ^{45}Ca influx into cells of the pheochromocytoma cell line PC12 is inhibited by nanomolar concentrations of nitrendipine.[114]

In whole animal studies, selectivity at the tissue level will also depend partly on various physiological feedback systems that will modify the effect of the Ca^{2+} channel antagonists. For example, any direct cardiodepressant effect of nifedipine that might occur with very high doses will be opposed by reflex autonomic nervous system effects (in response to hypotension), which increase heart rate and contractility. Decreased cardiac contractility due to Ca^{2+} channel inhibition will also be opposed by decreased resistance to blood flow in the whole body; the latter will allow increased cardiac stroke volume and total cardiac output even if the force of contraction is decreased. The low sensitivity of some but not other vascular beds in the intact animal is probably related to some extent to the relative importance in various types of local and autoregulation of blood flow, as well as regulation of flow by the nervous and endocrine systems. For example, vascular beds that are under a major and continuous regulation by the sympathetic nervous system, such as most of the cutaneous circulation, are often less sensitive to these vasodilators, whereas the coronary and cerebral circulation, which are more autonomous, are more susceptible to the vasodilator effect of Ca^{2+} channel antagonists.[35,52] This may reflect, in part, the norepinephrine levels that are generally present at the adrenergic receptors of a given vascular bed. It should be noted that the Ca^{2+} channel antagonists also have direct effects on baroreceptor reflexes and that these effects, such as the decrease in the cardiac parasympathetic component of the baroreceptor reflex, differ between the different drugs.[115]

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An example of frequency dependence of interaction and the effect of tissue pathology is seen in the use of verapamil to treat cardiac tachyarrhythmias. Therapeutic concentrations of verapamil do not significantly block cardiac Ca^{2+} channels until they are opened, and verapamil then slows the rate of recovery very dramatically. Therefore, the amount of block increases with heart rate. Thus, an abnormally high heart rate will be slowed more by verapamil than a normal heart rate. The amount of use dependence seen with nitrendipine (and presumably other 1,4-dihydropyridines) is much less than that with verapamil.[88]

It is clear that considerable variation occurs in the pharmacological sensitivity of Ca^{2+} channel events at the isolated cell levels. The extent to which this reflects differences in Ca^{2+} channel structure (presence, absence, or modification of antagonist binding sites), Ca^{2+} channel function (kinetics of activation and inactivation and their modification by membrane potential and antagonists), or differences in the coupling between channels and the effector components remains to be determined for each type of tissues. The recent availability of Ca^{2+} antagonist radioligands and binding assays does, however, permit further exploration of these possibilities.

(4) Sites and Mechanisms of Action. (a) Ligand Binding Studies with [^{3}H]Dihydropyridines. Although vascular smooth muscle is presently the major therapeutic site of action of nifedipine, nitrendipine, nimodipine, and related dihydropyridines, the first[116] and most extensive[74] characterization of binding sites for [^{3}H]dihydropyridines in smooth muscle was on membranes from intestinal smooth muscle. High-affinity binding ($K_d \simeq 0.1$ nM) of both [^{3}H]nitrendipine and [^{3}H]nimodipine[117] to membranes from ileum was found to be rapid, reversisible, specific, saturable, stereoselective, and of low density. The maximum number of binding sites was estimated to be 1–10 μm^{-2} of membrane surface area,[74] which is in good agreement with electrophysiological estimates of Ca^{2+} channel density in cardiac[118] and neuronal cells.[63,113] Radiation inactivation analysis gives a molecular weight of the binding site's multisubunit structure in smooth and cardiac muscle membranes of 275 000 daltons, which is similar to that of the Na^+ channel, and studies with a radiolabeled irreversible dihydropyridine derivative indicate a subunit molecular weight of 45 000 for the binding site.[120]

An important criterion for establishing that ligand binding is occurring at the pharmacologically relevant site is the demonstration that the same potency series is obtained for inhibition of binding and biological response. This has been done for membranes from guinea pig ileal smooth muscle, where there is an excellent correlation between the absolute potency of a variety of nifedipine analogues for inhibition of nitrendipine binding and for inhibition of ileal contractions induced by K^+ depolarizing media.[74] Binding data for membranes from bovine aorta,[121] as well as from rat,[117] rabbit,[122] and dog[123] ventricle, are essentially the same as that for guinea pig ileal smooth muscle, indicating a considerable similarity in these ligand recognition sites. Thus, for all these tissues, the correct potency series has been obtained and a marked enantiomer selectivity of interaction is observed.

The characteristics of ligand binding to membranes from guinea pig ileum[74,116,117] and bovine aorta[121,124] are consistent with the hypothesis that the binding site observed is that mediating the pharmacological effect of these compounds in these smooth muscles. A similar high-affinity binding site is also seen in membranes from a large variety of other smooth muscles[125–128] (see ref 129 for a more detailed discussion of ligand binding results for smooth-muscle membranes).

In contrast to the strong indications that the relevant pharmacological site is being studied in smooth-muscle membranes[74,116,121], doubt exists for similar binding sites in membranes from other sources.[122] Both cardiac and neuronal cells generally exhibit a low sensitivity to these drugs, but isolated membranes from these cells exhibit the same high-affinity binding that is seen in smooth muscle.[74,116,117,121–140] However, depolarization-induced ^{45}Ca uptake into pheochromocytoma PC12 cells[114] was blocked by nitrendipine at a low concentration ($IC_{50} = 10^{-9}$ M), which agrees with the dissociation constant for binding to isolated membranes from this clonal cell line ($K_d = 10^{-9}$ M). Initial studies with intact cultured cardiac cells indicate that both high ($K_d = 2.6 \times 10^{-10}$ M) and low ($K_d = 1.6 \times 10^{-8}$ M) affinity binding sites for [^{3}H]nitrendipine are present[141] and that the apparent affinity of the latter

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site also correlates with the EC_{50} for negative inotropic effects (2.8×10^{-8} M).

Studies of the effect of verapamil, D600, and diltiazem on 1,4-dihydropyridine binding have resulted in many conflicting reports.[128,130-134,136,138,143,144] However, there is now general agreement that verapamil decreases, whereas diltiazem increases, dihydropyridine binding, both apparently by allosteric mechamisms.[74,122,125,128,134,136,143,144] Of particular interest is a report by Murphy and co-workers,[134] indicating that all non-dihydropyridine Ca^{2+} channel antagonists (including bepridil, diltiazem, lidoflazine, prenylamine, and verapamil) appear to act at a common allosteric site to modify dihydropyridine binding to guinea pig brain membranes. Diltiazem appears to be a positive heterotropic regulator of the dihydropyridine binding site, whereas verapamil and D 600 are negative heterotropic regulators.

There are differences between tissues with regard to the effect of diltiazem on 1,4-dihydropyridine binding. In membranes from some tissues, for example, ileal smooth muscle and skeletal muscle, diltiazem increases the maximal number of binding sites without increasing the affinity of these sites,[74,122] whereas for swine coronary artery,[125] neuronal,[134,143,144] and rabbit ventricular membranes[122] an increase in the affinity, rather than a large increase in the maximal number of binding sites, is seen. This is of interest because it represents one of the few differences detected so far between membranes from different tissues in the binding sites for Ca^{2+} channel antagonists.

Another apparent difference between Ca^{2+} antagonist binding sites of membranes from different tissues is in the cation dependence of binding: EDTA treatment produces different effects on nitrendipine binding in membranes from brain, skeletal-muscle,[129,133] and smooth-muscle membranes.[74] The cation dependence of binding also represents another line of evidence that has been considered to support the idea that 1,4-dihydropyridine binding is to the Ca^{2+} channel[128,133,136-140] (see ref 122 for further discussion). After EDTA treatment, the amount of [^{3}H]nitrendipine binding to neuronal membranes is much less in the presence of Ba^{2+} than in the presence of Ca^{2+}.[133] If the number of binding sites is an index of the number of cation channels, then one might predict that less channels would be detected by using Ba^{2+} rather than Ca^{2+} as the current carrier, but this was not found to be the case.[113,118]

The apparent competitive antagonism between Ca^{2+} and Ca^{2+} channel antagonists (discussed above) observed in intact cells and tissues is not seen in [^{3}H]dihydropyridine binding assays over low (millimolar) Ca^{2+} concentrations; only at very high Ca^{2+} levels is there a reduction in drug binding, at which concentrations Mg^{2+} also inhibits.[128,133] Therefore, either Ca^{2+}–dihydropyrine binding site interaction is lost when membranes are isolated or the pharmacological antagonism is functional rather than competitive. In contrast, the binding of [^{3}H]verapamil to cardiac membranes has been reported to be antagonized by Ca^{2+} in the 4- to 9-mM range.[145] It remains to be determined whether this verapamil binding site is the same as the allosteric site that regulates dihydropyridine binding.

Other than known Ca^{2+} channel antagonists, most other compounds tested do not effect 1,4-dihydropyridine binding in low concentrations, indicating that this binding site exhibits a high degree of specificity. However, interesting exceptions have been found.[74,129] For example, diphenylhydantoin and diazoxide (10^{-4} M) both partially inhibit nitrendipine binding and both are thought to have a component of their pharmacological effect due to some type of Ca^{2+} antagonism.[146,147] Furthermore, certain antihistamines, neuroleptics, and anticholinergic agents have been found to produce diltiazem-like enhancement of nitrendipine binding (in the presence of tiapamil) by acting at the allosteric regulatory site.[134]

Autoradiography of brain slices indicates that the highest density of [^{3}H]nitrendipine binding sites is at synaptic areas rather than blood vessels.[148] These results are in agreement with the low numbers of binding studies found in vascular smooth-muscle membranes.[121,124-127,148]

Many studies with subcellular membrane fractions from smooth and cardiac muscle and nerve indicate that the localization of the specific binding site for Ca^{2+} channel antagonists is the plasma membrane,[74,121,123,125,126,133,135,137,138] although localization in cardiac terminal cisternae rather than sarcolemma has been proposed.[149] Most of the nitrendipine binding sites in skeletal muscle, which are of lower affinity than those of smooth or cardiac muscle or brain, appear to be in the transverse tubules rather than the surface sarcolemma;[150] reviewed in ref 151.

Most of these results, as well as the previously discussed electrophysiological studies, are in agreement with studies on skinned cardiac[39] and smooth muscle,[152,153] which collectively indicate that most Ca^{2+} channel antagonists have little or no effect in low concentrations on the intracellular membranes or on contractile or associated regulatory proteins. However, at high concentrations some of these drugs do bind to calmodulin,[154-156] as well as to calmodulin binding proteins, such as cyclic nucleotide phosphodiesterase,[156] and other studies also support the hypothesis that these compounds have multiple pharmacological actions.

(b) Do Ca^{2+} Channel Antagonists Have Other Mechanisms of Action? Many of the Ca^{2+} channel antagonists are very hydrophobic; therefore, high concentrations of these agents accumulate in cell membranes. Mean myocardial levels of verapamil exceed 10^{-6} M after intravenous injection of moderate doses (0.5 mg/kg) into dogs.[157] Other channel antagonists also accumulate in cardiac and smooth muscle cells so that their mean con-

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centration inside muscle cells may be 20-fold greater than the extracellular concentrations.[158] It is not known what contribution this higher concentration of drug in the membrane makes to therapeutic action, particularly after chronic treatment, but it is clear that Ca^{2+} channel antagonists have numerous actions that can be detected biochemically that are not related to blockade of Ca^{2+} channels and are in addition to the actions on calmodulin discussed above.

One such effect that occurs in relatively low concentrations is a marked stimulation of Na^+,K^+-ATPase of smooth-muscle (but not cardiac or neuronal) membranes by nimodipine and nitrendipine.[159] This stimulation is not due to the binding of nimodipine to the previously discussed high-affinity binding site,[117,160] since it is concentration dependent over a lower and wider range (10^{-9} to 10^{-6} M) and the number of Na^+,K^+-ATPase molecules in membranes are 1000-fold greater than the number of nitrendipine binding sites.[125] It is not known whether Na^+ pump stimulation occurs in intact cells, but the possibility of drug intervention at this site is exciting because inhibition of the Na^+ pump probably contributes to the etiology of essential hypertension (see ref 161 and 162 and references therein). Other evidence consistent with Na^+ pump stimulation by these drugs includes reduced intracellular Na^+ in blood vessels treated with diltiazem[54] and nifedipine;[163] however, these drugs produced little or no Na^+,K^+-ATPase stimulation, in contrast to nimodipine and nitrendipine. Other evidence for Na^+ pump stimulation by diltiazem has been previously discussed.[54] Hermsmeyer[164] has reported electrophysiological results on blood vessels that are consistent with electrogenic ion pump stimulation by nitrendipine, but he suggests that it is likely to be a Ca^{2+} rather than a Na^+ pump that is stimulated.

Ca^{2+}-ATPase and Ca^{2+} binding of skeletal and cardiac muscle sarcoplasmic reticulum are stimulated by extremely high concentrations (10^{-4} M) of nimodipine and nitrendipine, but not by nifedipine.[165] Previous workers have reported that D 600 and verapamil (10^{-5} to 10^{-4} M) inhibit Ca^{2+}-ATPase and Ca^{2+} transport by cardiac sarcoplasmic reticulum.[166-168] Verapamil and bepridil (10^{-6} to 10^{-5} M), but not nifedipine or diltiazem, inhibit Ca^{2+} binding to isolated cardiac sarcolemma, resulting in the suggestion that the mechanism for block of Ca^{2+} influx may vary between drugs.[169] However, verapamil blocks cardiac slow responses at lower concentrations than those at which it inhibits Ca^{2+} binding.[169] Furthermore, electrophysiological studies demonstrate that channel blockade does not result from displacement of Ca^{2+} that would permeate the channel, since verapamil, D 600, diltiazem, and nitrendipine block outward as well as inward Cs^+ current through the Ca^{2+} channel.[88]

Table VI. Effects of D600 and Verapamil Not Attributable to Block of Ca^{2+} Channels[a]

local anesthetic, Na^+ channel block	
inhibn of outward K^+ current (delayed rectifier, I_x)	
receptor blockade:	adrenergic α_1 and α_2
	muscarinic cholinergic
	opiate

[a] References 25, 34, 42, 55, 103, 110, 111, and 176-178.

Several studies indicate that low concentrations of these drugs have intracellular effects in cardiac cells. For example, bepridil produced more block of contraction than block of the slow inward current.[170] Similarly, verapamil, nifedipine, diltiazem, and perhexiline reduced the force of cardiac contraction more than intracellular Ca^{2+}, as estimated by an aequorin signal,[171] suggesting that they may act at the level of the contractile proteins; this view does not accord with the previous studies on skinned muscle discussed above. Other intracellular effects of these agents include inhibition of inorganic phosphate induced swelling of cardiac mitochondria[172] and inhibition of Na^+-induced Ca^{2+} release from this organelle.[173] The protective effect of diltiazem on the ischemic heart appears to be due not only to coronary vasodilation and decreased heart rate but also to other mechanisms.[174]

The above data consistent with ion pump stimulation in smooth muscle, as well as the intracellular effects in cardiac muscle, support previous suggestions[34,54,60,94] that these agents have sites of action in smooth muscle in addition to Ca^{2+} channels. In contrast, Cauvin et al.[50] have argued that the close correlation between blockade of ^{45}Ca influx and inhibition of smooth-muscle contraction (Table V) caused by various concentrations of diltiazem,[100] D 600, flunarizine,[92] nisoldipine,[50] and nitrendipine[175] establishes that the mechanism of action of these compounds in the tissues examined is exclusively blockade of stimulated Ca^{2+} influx.

Many of the above studies, along with electrophysiological investigations and ligand binding studies with labeled compounds for other receptor types, emphasize the dissimilarities between the pharmacological profiles of the various Ca^{2+} channel antagonists. Thus, verapamil and D 600 (in micromolar concentrations) block some Na^+ channels, K^+ channels, and a variety of receptor types (Table VI), whereas nifedipine and other 1,4-dihydropyridines appear to be relatively free of these actions.[176,178] Of particular interest are the results of Karliner and coworkers[177] indicating that verapamil blocks α_1 receptors

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and muscarinic receptors at concentrations (10^{-6} M) that are similar to those achieved clinically. The wide spectrum of pharmacological activity of verapamil and D 600 (Table VI) clearly indicates that these compounds are poor tools for examining the effect of Ca^{2+} channel blockade on a given cell type. In contrast to the marked stereoselectivity seen for verapamil and D 600 in blockade of smooth and cardiac muscle Ca^{2+} entry and contraction,[83-87] inhibition of Na^+ channels and neurotransmitter binding is not stereoselective.[87,179] Blockade of Na^+ channels, in contrast to the other effects on K^+ channels and other receptors (Table VI), occurs only in very high, nontherapeutic concentrations of verapamil. Unlike verapamil, tiapamil, dihydropyridines, and diltiazem, the antagonists lidoflazine and perhexiline block cardiac Na^+ channels in the same concentration range as they block Ca^{2+} channels.[180,181]

(c) Electrophysiological Studies. Direct measurements of Ca^{2+} currents in cardiac cells have been useful not only for establishing the basic site of action of these drugs but also for providing some exact information about their sites and mechanisms of action. Previous studies established that Ca^{2+} channels are blocked by verapamil and D 600 only after they are opened or inactivated and that the degree of block increases with membrane depolarization and frequency of stimulation.[84,89,182] The binding site for the Ca^{2+} channel antagonists appears to be modulated by the state of the channel in a manner analogous to the binding sites for local anesthetics in nerve[183] and cardiac muscle.[184] Nifedipine, like verapamil, exhibits frequency dependence in atrioventricular cells,[185] and nitrendipine shows use dependence in isolated ventricular cells.[88] Nitrendipine, in contrast to verapamil, D 600, and diltiazem, exhibits some resting-state block, whereas diltiazem may inhibit cardiac Ca^{2+} channels by binding mainly to the inactivated state.[88] Therefore, it might be expected that diltiazem would be less effective on Ca^{2+} channels that undergo little or no inactivation, such as those involved in stimulus-secretion coupling or those in skeletal muscle.[63] Kass[178] has suggested that the lack of a marked voltage dependence of block by nisoldipine may make this agent more useful than non-dihydropyridines in treatment of those cardiac arrhythmias not associated with abnormal depolarization. Recent studies with D 600 and its quaternary ammonium *N*-methyl derivative (D 890) indicate that D 600 blocks from inside of the Ca^{2+} channel after reaching its binding site by entering the cell in its unchanged form; D 890 was inactive unless it was injected inside of the isolated guinea pig myocyte.[186]

Summary and Future Prospects

Toward the beginning of this Perspective we posed a number of questions to be answered concerning the Ca^{2+} channel antagonists. Biochemical, chemical, clinical, pharmacological, and physiological studies collectively support the conclusion that this important group of molecules does function in specific fashion to inhibit Ca^{2+} channel function. Major questions of mechanisms and sites of action remain, however, to be resolved. The recent radioligand binding assay supports the conclusion, drawn earlier from the chemical and pharmacological heterogeneity of these agents, that there exists multiple sites and mechanisms of action for the Ca^{2+} channel antagonists. This is a satisfying conclusion, since, although it makes high demands on future experimentation designed to delineate these sites and mechanisms, it indicates the very real possibility for the development of tissue-selective Ca^{2+} channel antagonists. Elsewhere in this review we have already addressed the question of tissue selectivity as observed for existing compounds. In our opinion, the structural and pharmacological clues available should bring us closer to the goal of second- and third-generation Ca^{2+} antagonists with defined tissue selectivity.

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