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CALCIUM CHANNEL LIGANDS

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INTRODUCTION

The Ca^{2+} channel blockers, diltiazem, nifedipine, and verapamil, are now well-established members of the therapeutic armamentarium employed in cardiovascular disease including, but not limited to, angina in its several forms, hypertension, some cardiac arrhythmias including supraventricular tachycardia, congestive heart failure, and hypertrophic cardiomyopathy. Several reviews detail these and related clinical uses (1–8). The introduction of these agents into cardiovascular medicine has had several major and related consequences.

Investigators are examining the efficacy of Ca^{2+} blockers in a number of additional disorders both within and without the cardiovascular system, including achalasia, asthma, atherosclerosis, dysmenorrhea, intestinal spasm, labyrinthine disorders, migraine, peripheral vascular disorders, premature labor, and urinary incontinence.

In addition to diltiazem, nifedipine, and verapamil, a large number of related agents are coming into use or are under clinical investigation. In the 1,4-dihydropyridine (nifedipine) category these related agents include felodipine, nicardipine, nitrendipine, nisoldipine, nimodipine, and PN 200-110. Among the phenylalkylamine (verapamil) class they include gallopamil and tiapamil (Figure 1). As a consequence of this high level of clinical activity, discrete sections or chapters on Ca^{2+} channel antagonists are appearing in standard texts of pharmacology (9, 10).

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The Ca^{2+} channel blockers are drugs acting specifically at defined pathways of Ca^{2+} mobilization. This realization spurred considerable interest in their use as probes with which to characterize, isolate, and reconstitute Ca^{2+} channels (see 2, 4, 11–18 for reviews). The introduction of Ca^{2+} channel activators of the 1,4-dihydropyridine class, including Bay K 8644, provided a further stimulus (19–22, Figure 1). This latter development suggests *Ca^{2+} channel ligand* as a global term for drugs acting at Ca^{2+} channels.

Investigators recognized early that the Ca^{2+} channel antagonists were not uniformly effective in all tissues. They also realized that 1,4-dihydropyridines are smooth muscle selective (compared to the verapamil and diltiazem structures) and that neuronal tissues are, despite the presence of ligand-binding sites, frequently insensitive to these drugs. Such phenomena are now explained according to the concepts of state-dependent binding of Ca^{2+} channel ligands (23, 24) and of multiple classes of voltage-dependent Ca^{2+} channels that differ in their kinetic and permeation characteristics and in their pharmacological sensitivity (25). Additionally, introduction of the Ca^{2+} channel antagonists into both clinical and basic sciences focuses additional attention on the general regulatory role of Ca^{2+} in cell function (26).

The literature of the Ca^{2+} channel ligands has expanded dramatically in the

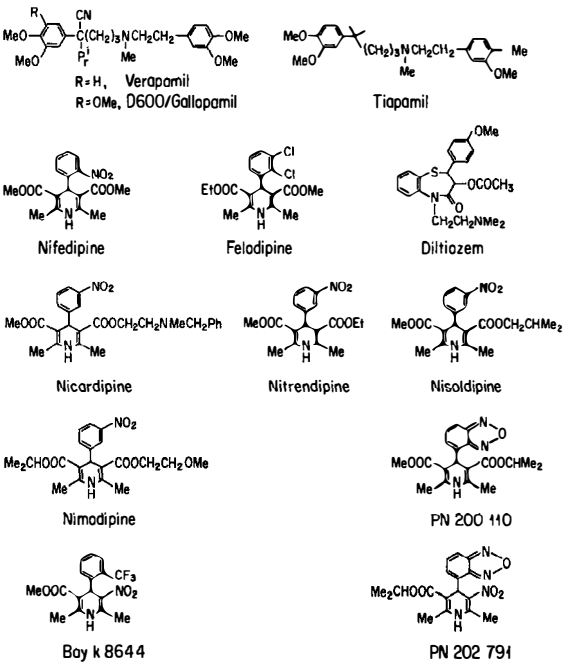

Figure 1 Structural formulas of Ca^{2+} channel ligands.

past several years. This review concentrates primarily on developments during 1985 and 1986 and refers only selectively to the earlier literature (27–30). It covers only the Ca^{2+} channels of the plasmalemma and associated structures and does not discuss the intracellular channels of the endoplasmic and sarcoplasmic structures. We organize this review around five main areas: (*a*) structure-function relationships; (*b*) properties and function of binding sites; (*c*) the basis of tissue selectivity; (*d*) regulation of Ca^{2+} channels; and (*e*) prospects for the future.

STRUCTURE–FUNCTION RELATIONSHIPS

Evidence for Specific Sites

The chemical and pharmacological heterogeneity of the major classes of Ca^{2+} channel ligands suggested that they interact at different sites and by different mechanisms to modulate Ca^{2+} currents. Consequently, no single all-embracing structure–function relationship could describe Ca^{2+} channel ligands (17). Radioligand-binding data validate this conclusion and generate a model for channel ligand binding of three discrete, allosterically linked binding sites for the major 1,4-dihydropyridine, phenylalkylamine, and benzothiazepine categories (See "Properties and Function of Binding Sites"; 11–14, 16). These linked interactions have been demonstrated in several pharmacological preparations (31–33). Structure–function relationships must therefore be interpreted in terms of interaction at these separate receptor sites.

Few significant advances in data accumulation for structure-activity relationships have occurred since the last comprehensive review (34), but developments in the 1,4-dihydropyridine field continue. Additional solid-state (35–37) and solution conformation (38) determinations are consistent with previous reports (39) that active 1,4-dihydropyridine molecules contain the substituted 4-phenyl ring positioned above and in the vertical plane of the 1,4-dihydropyridine ring, which itself is in a flattened boat conformation. Synthesis of rigid analogs in which the dihedral angle between the two rings is restricted confirms this proposal (40). Extensive comparisons of the pharmacological activities of a series of 1,4-dihydropyridines in smooth muscle, vascular and nonvascular (41, 42), confirm the previous observations (17, 43, 44) that despite quantitative tissue-dependent differences in activity the rank order is constant. This finding is consistent with the variable expression of a single structure–function relationship (See "The Basis of Tissue Selectivity"). Antibodies directed against 1,4-dihydropyridines show binding properties very similar to those exhibited by the membrane receptor, save for the absence of allosteric interactions with other structural categories of ligands and the independence of binding from divalent cations (45).

Orientational aspects of drug-receptor interactions are receiving increasing

attention. In principle, ligand approaches to ion channel extra- or intracellular binding sites may involve aqueous or membrane pathways. Such considerations are important with respect to both the rate (two- and three-dimensional diffusion; 46) and the extent (binding site availability) of the ligand-channel interaction. A model has been advanced for the 1,4-dihydropyridine interaction in which these hydrophobic molecules partition into the lipid bilayer and then diffuse laterally to a specific binding site (47, 48). If this model is correct, the implications for drug design may be quite important, since the partitioning and diffusion pathways may determine both ligand affinity and access to different channel states.

Antagonist and Activator Ligands

1,4-Dihydropyridine activators and antagonists differ only in minor structural aspects (Figure 1). Very limited structure-activity data are available for activators. The presence of a 3-nitro or 2,3-lactone substitution in the 1,4-dihydropyridine ring is important, but not sufficient, for significant activity. The solid-state structures of Bay K 8644 and CGP 28 392 are very similar to those for antagonist 1,4-dihydropyridines (49). The common conformational features of activators and antagonists are consistent with the suggestion that they interact at a common site (50–53). However, the enhanced 1,4-dihydropyridine ring planarity and acidity of the −NH proton, together with subtle differences in ester orientation in activator molecules, could contribute to the relative ability of 1,4-dihydropyridines to stabilize open and closed channel states (49). Of these electronic and structural differences, the most important may be the asymmetric disposition and function of binding sites for C_3 and C_5 substituents (49); this speculation is supported by the remarkable enantiomeric selectivity of Bay K 8644 and PN 202 791, where the S- and R-enantiomers are activator and antagonist, respectively (53–58). Binding of 1,4-dihydropyridines is dominated by hydrophobic interactions. In contrast to other receptor systems, including β-adrenoceptors (59), thermodynamic discrimination between activator and antagonist species was not observed (60).

Structure-Function Correlations

Radioligand-binding data from neuronal and skeletal muscle preparations yield structure-activity correlations very similar to those from cardiac and smooth muscle, despite the former studies' frequent absence of easily demonstrable pharmacologic effects (2, 16, 17). This absence is particularly obvious for neuronal tissue, where the binding data are virtually identical to those derived from functional preparations. Some binding sites may be nonfunctional or uncoupled (61, 62). However, resolution of this anomaly is probable, in part, through state dependence of ligand interaction, in which radioligand binding gains access to a state that is pharmacologically inaccess-

Table 1 Properties of major Ca^{2+} channel types[a]

	Persistent	Transient
Activation	− 10 mV	− 50 mV
Peak	+ 20 mV	− 10 mV
Tails	fast	slow
Inactivation	slow, incomplete	fast, complete
Permeation	$Ba^{2+}>Sr^{2+}>Ca^{2+}$	$Ca^{2+}\sim Sr^{2+}\sim Ba^{2+}$
Stability	labile	stable
$Metal^{2+}$	sensitive	less sensitive
Organic antagonists	sensitive[b]	insensitive or much less sensitive

[a]This table was compiled using data from various publications encompassing several tissue types. We do not intended to indicate that there are only two channel classes with precisely the characteristics defined above.

[b]Sensitivity of this channel type to 1,4-dihydropyridines is $\leq 10^{-9}$ M in smooth and cardiac muscle, but $\geq 10^{-6}$ M in many neuronal systems.

ible (See "The Basis of Tissue Selectivity"). The existence of different Ca^{2+}-channel classes with different pharmacological specificity may also aid this understanding. Accordingly, quantitatively and qualitatively different structure–activity relationships should exist. Examples of both are known. Quantitative variations in the expression of a structure–activity relationship are consistent with state-dependent interactions (See "The Basis of Tissue Selectivity"; 41–44), and considerable electrophysiological data document the existence of at least two kinetically and pharmacologically distinct voltage-dependent Ca^{2+} channels (Table 1) in neurons (63–67), cardiac, skeletal and smooth muscle (68–71), and secretory cells (72).

The recognition of distinct Ca^{2+}-channel types and processes underlies the search for new structures and the possible reevaluation of existing activities. Comparatively little information is available currently, but the area is ripe for rapid development.

New Channel Ligands

Some of the actions of benzodiazepines have been interpreted in terms of Ca^{2+} channel modulation, but whether these effects are primary or secondary remains to be determined (73). Peripheral, rather than central, benzodiazepine ligands appear to be the more active, although the function of the peripheral site is unknown and appears physically distinct from the 1,4-dihydropyridine-sensitive Ca^{2+} channel (74, 75). Insufficient data are available to establish whether benzodiazepines are selective for a particular channel class. The peripheral ligand PK 11195 has been suggested to function by stabilizing the resting state of the 1,4-dihydropyridine-sensitive channel and thus may serve to antagonize both Ca^{2+}-channel antagonists and activators (76, 77).

Organization of pharmacological and structural knowledge of the Na^+ channel was greatly assisted by the existence of specific toxins. Such toxins and related materials for the Ca^{2+} channel are becoming available (78). They include the dinoflagellate toxin, maitotoxin (79, 80); atrotoxin from *Crotalus atrox* venom (81); β-leptinotarsin-h from the hemolymph of the beetle *Leptinotarsa haldemani* (82); and w-conotoxin GVIA, a toxin from fish-eating molluscs of the *Conus* genus (83, 84). The conotoxins affect several ion channels and receptors. GVIA, with 27 amino acids, is of particular interest. It blocks presynaptic Ca^{2+} channels with a pharmacological and radioligand-binding profile that distinguishes it from the sites controlled by the dihydropyridine, verapamil, and diltiazem class of ligands (85, 86). This toxin probably defines the *L*- and N-channels of neurons, rather than the long-lasting 1,4-dihydropyridine-sensitive *L*-channels that appear currently to dominate the cardiovascular system.

Voltage-dependent Ca^{2+} channels are modulated by a number of neurotransmitters and neuropeptides including catecholamines, acetylcholine, histamine, serotonin, adenosine, somatostatin, and opiates (28, 87). The existence of both antagonist and activator 1,4-dihydropyridine ligands (88) raises questions concerning the existence of endogenous regulators and the physiological basis of excitatory and inhibitory ligand control of Ca^{2+} channels. Thus, the central issue becomes communication between receptor and channel: how are these macromolecules linked? Phosphorylation through the cAMP-dependent protein kinase plays an indirect role in control (89). However, recent evidence indicates that guanine nucleotide–binding (G) proteins are involved in both turning on and turning off Ca^{2+} channels (90–92). One possible explanation is that the G proteins couple directly channels and receptors. Such membrane organization would have important implications to both Ca^{2+} channel organization and the definition of structure–activity relationships for ligand modulation of Ca^{2+} channels.

PROPERTIES AND FUNCTION OF BINDING SITES

General Properties

The characteristics of the sites for nonpeptide Ca^{2+} channel ligands, as revealed by radioligand-binding studies, have been reviewed several times (11–14, 16–18, 29, 93). Quite generally, membranes from different tissues have very similar affinities for 1,4-dihydropyridine ligands ($\sim 10^{-10}$M for nitrendipine), except for skeletal muscle, which has an approximately 10-fold lower affinity. These affinities are all similarly temperature dependent over the temperature range 0–37°C, consistent with an identity or near identity of the 1,4-dihydropyridine binding site in several preparations (60). The

relationship of this site to the permeation machinery of the channel remains unknown (94). The thermodynamic data are consistent with a dominantly hydrophobic interaction of the ligands; activators and antagonists were not distinguished, perhaps reflecting ligand binding to a depolarized, inactivated channel state. Recent size and immunoreactivity studies (95) support the finding that the recognition site of the 1,4-dihydropyridine receptor is very similar in different tissues. The densities of binding sites for 1,4-dihydropyridines in purified or partially purified membranes are: skeletal muscle t-tubules (> 65 pmol/mg), cardiac sarcolemma (1–2 pmol/mg), synaptosomal membranes (0.6 pmol/mg), nonvascular and vascular smooth muscle sarcolemma (0.8 and 0.2 pmol/mg, respectively). Vascular smooth muscle, the major target tissue for nifedipine and related drugs, appears to have the lowest density of sites for these drugs (11, 12, 16). Studies of single channel conductances (96) confirm that differences exist between Ca^{2+} channels of similar pharmacological sensitivity.

1,4-Dihydropyridine receptors are susceptible to proteases, phospholipases, -SH reagents, heat, and divalent cations (11–14, 93). Despite the previously mentioned similarities between receptors in different tissues, skeletal muscle binding sites do appear to be biochemically distinguishable by several criteria, including susceptibility to EDTA, -SH reagents, and 1,4-dihydropyridines (93). The receptors in neuronal membranes may differ from those in heart in their sensitivity to monovalent and divalent cations (97). These results indicate that receptors in different tissues may exhibit differences in their associated regulatory sites for cations and other substances.

Purification on lectin columns shows these receptors to be glycoproteins. The high-affinity binding site of the 1,4-dihydropyridine receptor is also susceptible to voltage, as both electrophysiological and ligand-binding studies show (98; See "The Basis of Tissue Selectivity"). The voltage-dependent interactions, together with the correlations between pharmacology and binding and the reconstitution experiments, provide strong evidence for the association between high-affinity sites for 1,4-dihydropyridines and Ca^{2+} channels.

The binding of each of the main classes of Ca^{2+} channel ligands to membranes from excitable cells, except for those in skeletal muscle, is dependent on micromolar concentrations of divalent cations (11–14, 16, 93). In addition, millimolar concentrations of extracellular Ca^{2+} inhibit the binding of verapamil- and diltiazem-like ligands (99). Whether the high-affinity divalent cation–binding sites within the Ca^{2+} channel (44) are the same sites required for the binding of these drugs remains to be determined. Ca^{2+} produces Ca^{2+}-dependent inactivation at a site that is unlikely to promote the binding of these drugs, since Mg^{2+} also promotes drug binding but does not cause channel inactivation.

Localization

Studies with [^{3}H]nitrendipine suggest very strongly that the high-affinity binding sites for this drug are associated with Ca^{2+} channels in the plasma membrane and its invaginations in excitable cells (11–14, 16, 93). Electrophysiological studies using dialyzed cells confirm this localization (100). Reconstitution of purified skeletal muscle t-tubular membranes (96, 101), solubilized Ca^{2+} channel protein (102), and cardiac sarcolemma (96, 103) further establish the sarcolemmal localization of these binding sites.

Both ligand-binding and autoradiographic studies (104, 105) show that high-affinity binding sites for 1,4-dihydropyridines exist in synaptic areas of the brain. Behavioral (106, 107) and biochemical (66, 108) studies indicate that the binding sites for Bay K 8644, and therefore other 1,4-dihydropyridines, represent functional sites on neurons. Central effects of Ca^{2+} channel antagonists are also seen, but usually only under special conditions, presumably those needed to produce a channel state that permits antagonist interaction. Collectively these results indicate that at least some of these high-affinity binding sites in the brain are on Ca^{2+} channels (16, 17, 66, 107, 108).

Electrophysiological studies reveal large numbers of Ca^{2+} channels in skeletal muscle, which is consistent with the high binding-site density of t-tubules (11–14, 16). However, electrophysiological estimates of functional Ca^{2+} channel density suggest that if these sites are on Ca^{2+} channels, then most may not represent functional channels (62). The finding that only 2–3% of purified 1,4-dihydropyridine-binding site–channel complex was reconstituted into functional channels is consistent with this hypothesis (102). Most of the 1,4-dihydropyridine-binding sites in skeletal muscle may be located on the abundant voltage-sensing sites involved in coupling t-tubule excitation to Ca^{2+} release from the sarcoplasmic reticulum (109).

Correlation Between Ligand Binding and Function

Many studies with smooth and cardiac muscle indicate that high-affinity binding of 1,4-dihydropyridines is that to Ca^{2+} channels. The correlation of the potencies of these drugs for inhibition of 1,4-dihydropyridine binding and for pharmacological response provided the first direct evidence for this view (10–12, 16–18). Recent reports extend these observations to a variety of smooth muscles (41, 42). The correlations obtained suggested that the same Ca^{2+} channels were activated in the different smooth muscles studied, regardless of the nature of stimulant (K^+ depolarization or specific agonist). These correlations also extend to the allosteric interactions between ligands of different structural categories that are seen in both binding and pharmacological experiments (31–33). Similarly, ligand-binding studies suggesting that

no direct competition occurs between Ca^{2+} and 1,4-dihydropyridines were confirmed electrophysiologically (110).

The various reconstitution experiments mentioned above also support the view that the ligand-binding sites are associated with Ca^{2+} channels. Unfortunately, in the only case in which purified proteins were reconstituted into vesicles, the degree of incorporation was poor (102). Also, as already noted, evidence shows that for cells in skeletal muscle, most of the binding may be to a protein, the voltage sensor. The voltage sensor may be closely related to, but is not itself, the Ca^{2+} channel (109).

Studies on neuroleptics and antidiarrheal agents (104) show that ligand binding can be used to predict effects on Ca^{2+} channels. To date no drugs inhibit 1,4-dihydropyridine binding without affecting Ca^{2+} channels (17).

There are at least three distinguishable sites for Ca^{2+} channel ligands, one each for 1,4-dihydropyridine, verapamil, and diltiazem analogs. Several studies on purified membranes from skeletal and cardiac muscle (99, 103, 111) indicate that the ratio of these binding sites is 1:1:1. Less-pure membranes yield different stoichiometries (13, 112). Other workers suggest that diltiazem and verapamil share common, or have overlapping, binding sites (104, 112). The differences to pH, temperature, -SH reagents, and 1,4-dihydropyridines (99) in the sensitivity of binding of these two ligand classes make this suggestion unlikely.

The binding sites are allosterically coupled such that at 37°C 1,4-dihydropyridine antagonists and diltiazem reciprocally stimulate binding (11–14, 16, 99, 111). Verapamil (and phenylalkylamines) and 1,4-dihydropyridines are mutually inhibitory independent of temperature (99, 111). Solubilized membranes from cardiac (113) and skeletal muscle (13, 14) also display these allosteric interactions. Diltiazem and verapamil also show mutual inhibition of binding. In contrast to the effects of 1,4-dihydropyridine antagonists, activators such as Bay K 8644 inhibit diltiazem binding in heart membranes (99) and stimulate it in brain membranes, but only at high concentrations (114).

At present these studies of ligand interactions present a complex picture. Transitions probably occur between different states of each receptor during ligand binding and a preexisting equilibrium likely exists between different affinity states of each binding site (115). Interpretations of radioligand-binding data are complicated by three realizations: that the membrane studies may reflect a dominantly inactivated channel state, that chiral 1,4-dihydropyridines may generate enantiomeric-specific activation and antagonism, and that one identified low-affinity 1,4-dihydropyridine binding site represents the adenosine transporter (116, 117). Similarly, the low-affinity phenylalkylamine binding site present in sarcoplasmic reticulum may not be related to the high-affinity Ca^{2+} channel component (118).

Isolation

Several laboratories have reported the isolation of the skeletal muscle 1,4-dihydropyridine t-tubule channel complex, and its reconstitution from the solubilized protein has been reported (102). In this latter preparation, subunits of 135, 50, and 33 kd were reported, but others have not detected the 50-kd component (95). The large 140-kd glycoprotein is bound to the smaller subunit (33 kd) by disulfide bridges (170 kd; 95). 1,4-Dihydropyridine receptors from cardiac and smooth muscle appear composed of 135- and 33-kd subunits. Antisera to the small subunit from skeletal muscle reacted with the small or large components from smooth and cardiac muscle under reducing or nonreducing conditions, respectively. Thus, the size and immunohistochemical characteristics in the three types of muscle appear very similar. Other immunological (45) and genetic (119) approaches are likely to be extremely useful for further channel purification and characterization.

THE BASIS OF TISSUE SELECTIVITY

Ca^{2+} channel antagonists show significant tissue selectivity both pharmacologically and therapeutically. In principle such selectivity may have its origin in the following factors, either alone or in combination:

1. Pharmacokinetic factors: tissue distribution and time course;
2. Ca^{2+} source mobilized: relative use of intra- and extracellular pools;
3. Ca^{2+} channel class: different pharmacological profile of channel classes;
4. State-dependent interactions: affinity of drug depends on channel state;
5. Nonspecific effects: ability to interact at other sites (receptors, etc); and
6. Pathologic state: organ or tissue state may influence above factors.

All of the preceding factors may be important, depending upon circumstance, but recent investigations focus on the existence of discrete channel categories with differing pharmacological sensitivities (See "Structure-Activity Relationships") and on the role of state-dependent phenomena in defining Ca^{2+} channel–ligand interactions.

Ligand affinity for channels may vary dramatically between resting, open, and inactivated states, according to stimulus frequency and membrane potential (23, 24, 28, 30). Use-dependent blockade, in which the inhibitory activity of a drug increases with increasing frequency of stimulation, reflects a preferential ligand interaction with, or access to, an activated or inactivated channel state. This occurrence has been described for verapamil, diltiazem, and their analogs (for reviews see 30, 120). These use-dependent actions are also influenced by the level of the membrane potential between stimuli -hyperpolarization and depolarization reducing and increasing, respectively, the extent of use-dependent blockade.

Until recently the 1,4-dihydropyridines were assumed to behave very differently and to exhibit little, if any, state-dependent interaction (see, for example, 121, 122). However, both activator and antagonist 1,4-dihydropyridines show significant voltage-dependent and, under the appropriate circumstances, frequency-dependent interactions (57, 71, 98, 110, 123–129). Original studies by Sanguinetti & Kass (124, 125) and by Bean (126) indicated that 1,4-dihydropyridine block of cardiac Ca^{2+} channels was strongly enhanced by membrane depolarization, was frequency dependent at pulse frequencies greater than 1 Hz, and occurred preferentially at inactivated Ca^{2+} channel states. Indeed, the high-affinity interactions occurring in depolarized preparations, $K_D \sim 10^{-9}$–10^{-10} M, are very similar to those derived from radioligand-binding experiments in membrane preparations. The significantly lower affinities observed in polarized preparations, $K_D \sim 10^{-7}$–10^{-6} M, contribute in large part to the observed discrepancies between 1,4-dihydropyridine binding and pharmacological actions in cardiac cells (11, 12, 16, 17, 29). The 1,4-dihydropyridine block of Ca^{2+} currents is enhanced by depolarizing prepulses and relieved by hyperpolarization, as subsequent investigations show (57, 71, 98, 127–129). Thus, 1,4-dihydropyridines interact with differing affinities at the resting, open, and inactivated states of the Ca^{2+} channel in a structure-dependent fashion. Nicardipine, which differs from other 1,4-dihydropyridines by the presence of a basic amine function, shows both the frequency-dependence characteristics of verapamil and diltiazem and the potential-dependence characteristics of 1,4-dihydropyridines (125).

The extent to which 1,4-dihydropyridines stabilize different channel states determines the activator-antagonist properties of this ligand series. Thus, Hess et al (77) indicate that Ca^{2+} channels possess three basic gating modes, 0, 1, and 2, which are characterized by no openings because of channel unavailability, brief openings, and long-lasting openings that appear rarely, respectively. 1,4-Dihydropyridine antagonists favor state 0; activators favor state 2. 1,4-Dihydropyridine ligand interaction with, and discrimination between, Ca^{2+} channel states exhibits many interesting subtleties and complexities. The previously noted enantiomeric selectivity of chiral 1,4-dihydropyridines (54–58) presumably reflects a configurational change in the several channel states. However, whether a single 1,4-dihydropyridine enantiomer can exhibit both activation and antagonism is not yet resolved. Tension studies in smooth and cardiac muscle (55, 58) and current studies in myocytes (130) show that the (−)-activator isomer of Bay K 8644 is both an activator and antagonist, according to membrane potential, whereas the (+)-enantiomer is solely antagonistic. However, in pituitary cells, where the Ca^{2+} channels are very slowly inactivated, (−)Bay K 8644 exhibited activator properties only (131). These state-dependent ligand interactions depend both on the relative availability of the different channel states and on the relative

ligand affinities for these states (77, 110, 129). Accordingly, the expression of activator and/or antagonist properties of a 1,4-dihydropyridine depends on the one hand on the chemical and configurational properties of the ligand and, on the other hand, on channel characteristics and channel-state dominance.

The preceding considerations suggest that discrete affinity states should therefore be accessible to radioligand-binding experiments. Although both high- and low-affinity sites have been detected for 1,4-dihydropyridine and verapamil ligands (11–14, 16, 17, 98, 112, 132–135), many studies attempting to demonstrate state (voltage)-dependent binding have serious limitations. Nonspecific binding levels may be unacceptably high at the concentrations necessary to detect low-affinity binding. Many studies have been carried out in depolarized (inactivated) membrane fragments, and the low-affinity sites described have not been pharmacologically characterized. These sites could represent other sites such as the adenosine transporter (116, 117), or they could represent intracellular sites of unknown significance (99, 112, 135). However, a component of voltage-dependent [^{3}H]nitrendipine binding that is lost upon hyperpolarization has been detected in cardiac sarcolemmal vesicles (134). In cultured cardiac cells PN 200–110 binding is voltage dependent, with K_D values of 0.73×10^{-9} M and 0.06×10^{-9} M at -40 and 0 mV, respectively (98). These and similar data obtained in competition studies (98), although not revealing K_D differences of the magnitude expected from electrophysiological observations, are nonetheless encouraging. They suggest a radioligand-binding correlate to state-dependent ligand interactions at the Ca^{2+} channel. Importantly, differential access, rather than differential binding, may also underlie state-dependent interactions (136). Other factors besides these several listed factors may contribute to tissue selectivity of the Ca^{2+} channel ligands. One such factor is the distribution of different channel types at both the organ and cellular levels. It will be important to determine how channel types are distributed between nodal and nonnodal regions in cardiac tissue and between cell bodies, dendrites, and terminals in neurons.

REGULATION

The availability of ligands for at least two categories of voltage-dependent Ca^{2+} channels makes it possible to determine the conditions, mechanisms, and consequences of the regulation of Ca^{2+} channels and the associated drug binding sites under physiological and pathological conditions. How closely such regulation follows the patterns established for other membrane effectors remains to be determined (137). Most studies to date focus only on radioligand-binding changes; correlates to functional channel changes are needed.

The cardiovascular crises that may follow abrupt clonidine or propranolol

withdrawal are well documented (138, 139). Several clinical reports are now available concerning Ca^{2+} channel antagonist withdrawal (140–147). Possible withdrawal symptoms were reported in some (140–143, 146), but not other (144, 145, 148), studies, following abrupt termination of diltiazem, verapamil, or nifedipine therapy. Tachyphylaxis to verapamil was reported in one case (148). Reactivity studies in rats chemically treated with D600 (gallopamil, methoxyverapamil) or in humans following nifedipine or verapamil treatment (149, 150) indicate a hyperresponsiveness that may reflect a compensatory effect. With the exception of a report on verapamil (143), an objective withdrawal phenomenon occurs in very few patients (144). Binding studies are inconclusive. 1,4-Dihydropyridine binding site density is unaffected or reduced in heart (151, 152) and reduced in brain (151, 153) following chronic treatment with nifedipine or verapamil. Exposure to Bay K 8644 produces both down-regulation (high dose) and up-regulation (low dose) of cardiac 1,4-dihydropyridine binding sites (P. Gengo & D. J. Triggle, unpublished data).

These reports represent initial attempts to define the channel regulation mediated by Ca^{2+} channel ligands. Such channel regulation may differ from that of membrane receptors for hormones and neurotransmitters because such receptors are tonically regulated by endogenous receptor-directed signals. Endogenous signals for the Ca^{2+} channel may be Ca^{2+} itself, endogenous factors presently unknown, or the input from associated activating or inhibitory receptors. Channel traffic and intracellular Ca^{2+} accumulation is one component of Ca^{2+} channel inactivation (154). Epidemiological and other studies (155, 156) suggest that reduced serum Ca^{2+} levels may be associated with hypertension. Whether chronic changes in Ca^{2+} levels are reflected in changes in Ca^{2+} channels or in other specific components of the Ca^{2+} regulatory machinery of the cell is not known.

It is not clear whether, and under what conditions, Ca^{2+} channels and membrane receptors may coregulate. In rats, chronic antagonist and agonist treatment, which up- and down-regulates β-adrenoceptors and muscarinic receptors, respectively, did not affect 1,4-dihydropyridine-binding-site density (157). However, denervation by reserpine or 6-hydroxydopamine increases both cardiac β-adrenoceptors and Ca^{2+} channels (158, 159). This finding suggests a role for neuronal influences on channel ligand–binding sites other than, or additional to, the tonic sympathetic activity. Consistent with this suggestion, nitrendipine-binding-site density is higher in cultured chick heart cells than in similarly aged cells from functioning hearts (160). Cardiac tissue from patients chronically treated with β-blockers or Ca^{2+} channel antagonists contains elevated β-adrenoceptor density (161), suggesting coregulation. Coregulation of Ca^{2+} channels and receptors, if it indeed occurs, may be uni- or bidirectional and may involve indirect or direct

linkages between the two membrane components. Thus, in skeletal muscle (162) and neuronal cell lines (163), elevation of cellular c-AMP levels by various agents is associated with increases in or the appearance of 1,4-dihydropyridine binding sites. c-AMP-dependent protein kinase–mediated phosphorylation is associated with Ca^{2+} channel activation in excitable tissues (164, 165); it may also be associated with Ca^{2+} channel expression. Channels and receptors may also have shared components through which communication occurs. A guanine nucleotide–binding (G) protein is (as measured by the effect of nucleotides and pertussis toxin) involved in the coupling of inhibitory norepinephrine and GABA signals to Ca^{2+} channels in dorsal root ganglia (90, 91). G proteins may couple directly receptors and channels.

Ca^{2+} channels are also regulated under pathological conditions. At least two diseases described are associated with alterations in Ca^{2+} channel ligand binding. Embryonic muscular dysgenesis in mice is characterized by disorganized triadic structure and decreased 1,4-dihydropyridine-binding sites in skeletal muscle; cardiac muscle was not affected. These changes could be directly linked to the observed defect in excitation-contraction coupling (166). The Syrian cardiomyopathic hamster has been reported to have higher densities of 1,4-dihydropyridine-binding sites in muscle and brain (167). This change may underline both the pathology, calcium-induced necrosis, and the therapeutic efficacy of Ca^{2+}-channel antagonists in this condition and in human hypertrophic obstructive cardiomyopathy. However, others have not found evidence for alterations in cardiac 1,4-dihydropyridine binding sites in this animal model (167a). Both elevated and reduced [^{3}H]nitrendipine binding have been reported in brains of DOCA- and spontaneously hypertensive rats (168, 169); how significant, if at all, these changes are to hypertension remains to be defined. A reduced dietary Na^+ intake increases nitrendipine-binding-site density in adrenal glomerulosa cell membranes, but is without effect in vascular and nonvascular smooth muscle (170). Thus, enhanced glomerulosa cell sensitivity to angiotensin during Na^+ reduction may in part reflect the increased number of Ca^{2+} channels as well as the increased number of angiotensin receptors.

Further studies demonstrating channel changes with physiological and pathological events will undoubtedly be forthcoming. Currently, many studies are based on equating changes in ligand-binding properties with changes in channel function. This assumption may not be unreasonable. However, correlative studies are needed, since binding and function are not necessarily coregulated (171). Ontogenic (171) and aging (172, 173) studies have described binding site changes, but have not measured function. This limitation is very important, since studies of the development of 1,4-dihydropyridine

binding sites and of functional Ca^{2+} channels in chick heart show clearly that the two events are temporally distinct (160) and that nonfunctional or uncoupled binding sites exist early in development.

PROSPECTS FOR THE FUTURE

In the past few years particularly dramatic progress has been made in the area of Ca^{2+} channel drugs. The introduction of these drugs into clinical medicine, the subsequent development of the radioligand-binding assay, and the electrophysiological assault on the Ca^{2+} channel have greatly contributed to this progress. The recent introduction of Ca^{2+} channel activators spurred more research in an already active field.

Currently, most research is centered on classifying Ca^{2+} channel categories by biochemical and electrophysiological techniques. Undoubtedly in the next few years major developments will occur in the areas of new channel ligands, genetic analyses of channel structure and function, development of new therapeutic areas (including particularly neuronal sites), and identification of Ca^{2+} channel defects with human disease states. The recent identification of Ca^{2+} channel ligand-binding sites in plants raises important questions about the functions and possible exploitation of these sites (174, 175).

Increasing pharmacological awareness of the spectrum of events, both specific and nonspecific, modulated by the Ca^{2+} channel ligands will also serve to focus attention on areas other than Ca^{2+} channel modulation. Some categories of Ca^{2+} channel blockers, notably verapamil, lower the resistance of malignant cells to a number of antitumor agents. This action is receiving considerable attention, although it is probably unrelated to channel blockade (176). Similarly, we may expect continued elucidation of the roles of Ca^{2+} channel ligands in cell protection and of the relationship of the observed protection to channel blockade (177–179). Finally, the significance of Ca^{2+} channel blockade to the development of atherosclerotic lesions and tissue calcification (2, 180–182) requires further investigation to determine the cellular and molecular basis of the observed protection.

The exploration of these future directions makes it very probable that Ca^{2+} channel ligands will enjoy prominent pharmacological and therapeutic roles for many years to come. Patient and scientist alike will benefit from this progress.

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