

III. Classification of Calcium Channels and the Sites of Action of Drugs Modifying Channel Function

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I. Introduction

IN this paper, we present a classification of calcium channels and drugs acting at these channels, with the aim of describing a prospective classification that will allow for changes as new channels or drug-binding sites are established. This report represents the consensus view of the calcium channel subcommittee (table I) of the International Union of Pharmacology receptor nomenclature committee. The classification is not a comprehensive documentation of the literature but reflects the current state of accepted knowledge, with criteria to allow classification of channels and sites of action of drugs modifying channel function. The classification is based primarily on channels, the many intracellular sites for Ca²⁺ await further attention.

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It must be emphasised that there may be several differences in the way compounds interact with ion channels compared with more classically defined receptors such as adrenoceptors, and the concepts used in this classification differ in some respects from those used by some of the other receptor classification committees. VDCCs have distinct binding sites for many drugs, but there may be no endogenous ligands for these sites. Although the predominant control of channel function may be by voltage changes across the membrane, the sensitivity to voltage may be modified by many factors such as channel phosphorylation, binding of G proteins to the channel, or binding of compounds such as DHPs.* Such changes might change sensitivity to membrane voltage to such an extent that activation may fall within the range of the resting membrane potential of a given

* Abbreviations: DHP, dihydropyridine; VDCC, voltage-dependent calcium channel.

TABLE 1

Composition of International Union of Pharmacology subcommittee on classification of calcium channels and the sites of action of drugs modifying channel function

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cell and voltage control is lost under physiological conditions. Nevertheless, the structure may still be a voltage-dependent channel. Channels may also exist with different conformational states (i.e., closed, open, inactivated), each state having different affinities for different drugs. Thus, interventions that change the proportion of the different channel states may modulate the sensitivity of the channels to these drugs, and, if the drugs vary in their affinities for the different states, these changes may be complex. This means that it is dangerous to use rank orders of potencies of inhibitors to assess channel types (as has been used with agonists in receptor classification), because the same channel may give different rank orders of potency depending on the preponderance of the different states. Therefore, we list the appropriate criteria for classification of channels and of binding sites on the channels.

II. Historical Aspects

The importance of Ca^{2+} for the maintenance of myocardial contractility was observed by Ringer as early as the 1880s, but only in the last three decades has the critical role of Ca^{2+} in the contractile processes been established in skeletal, cardiac, and smooth muscle. Ca^{2+} plays a crucial role in secretory responses and is perhaps the most widespread second messenger in eucaryotic cells.

During the 1960s, the concept of drugs acting as "calcium antagonists" was put forward by the research groups of Fleckenstein and Godfraind. Fleckenstein's group discovered the highly selective "calcium antagonistic" action of verapamil and its methoxy derivative D-600 (gallopamil), which showed interference with Ca^{2+} -dependent excitation-contraction coupling in the myocardium without an inhibitory influence on the Na^+ -dependent parameters of the action potential. He later added nifedipine and diltiazem to this group of drugs. The effects of the drugs could be overcome by increasing the extracellular Ca^{2+} concentration or by agents that could improve the availability of Ca^{2+} to the contractile system such as β -adrenoceptor agonists or cardiac glycosides. Godfraind's group made a major contribution to the development of the concept in the smooth muscle, showing that diphenylpiperazine analogues such as cinnarizine inhibited agonist-evoked contractions dependent on extracellular Ca^{2+} and defining the interrelationship of the inhibitory effects of the drugs with K^+ and Ca^{2+} . The concept of "calcium antagonism" was later extended to many other drugs including many diphenylpiperazine derivatives and the wide range of DHP derivatives of which nifedipine was the prototype. The historical development of the concept has been extensively reviewed (Fleckenstein, 1983a,b; Godfraind et al., 1986). In radioligand-binding studies, from 1982, it became clear that different binding sites existed for the drugs. Several classifications were proposed by individual

workers (Fleckenstein, 1988; Ferry and Glossmann, 1982; Godfraind et al., 1986; Spedding, 1982, 1985a,b; Janis et al., 1987; Nayler, 1988), by an international committee (Vanhoutte and Paoletti, 1987), and later by a cardiology working group (Opie et al., 1987). The classification of Vanhoutte and Paoletti was based primarily on functional differences between the drugs, with the aim of providing a rationale for the treatment of different clinical indications.

Recently, further evidence has accrued showing that many different forms of Ca^{2+} -selective channels may exist, some of which may be receptor gated. Furthermore, the primary structures of some forms of Ca^{2+} channels have been elucidated, and the sites of action of drugs modifying channel function are being defined. The classes of ion channel and the binding sites for drugs on these channels have been revisited and criteria established for their classification.

III. Criteria for Classification

A. Channel Classification

The following techniques should be used to define different channels and the site of action of drugs.

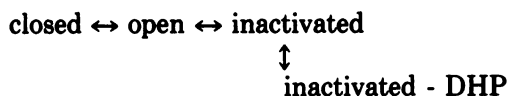
1. *Functional studies.* Functional studies are crucial to define mode of action and selectivity in a more physiological milieu. Definition of channels should be considered using different criteria from those traditionally associated with receptor classification (i.e., agonist and antagonist potencies). Because some drugs may have affinity for only certain states of the channel, affinity may change depending on the activation conditions used, engendering different rank orders of potency under different experimental conditions (see below). Rank order of potency of different agents is, therefore, insufficient to define channel subtypes because the potency of some compounds may be more susceptible than others to these phenomena. Drug interaction studies (competition experiments with drugs sharing the same binding site, reversal with channel activators, noncompetitive interactions with allosterically linked sites) should confirm the site of action. An important criterion for selectivity of action is to establish that forms of cellular activation that do not utilise the ion channel in question are not modified by the drug.

Determination of the affinity of drugs for ion channels may be critically dependent on the use and voltage dependency of the drug, which is dependent on the binding site. At sodium channels, binding of tetrodotoxin is relatively non-voltage dependent, and affinity for sodium channels is, consequently, easy to quantify. This contrasts markedly with the interaction of DHPs with L-type channels. Although it is now accepted that DHPs usually have high affinity for the inactivated state of the channel and low affinity for other states (closed, open), they may have high affinity for the open state in smooth muscle cells (Cohen and McCarthy, 1989). Consequently,

there will be an affinity constant for a high-affinity state, which is close to the high-affinity constant (usually less than nanomolar) observed in binding experiments. In functional experiments, however, the *proportion* of channels in the high-affinity and low-affinity states will critically modify the apparent affinity of the DHP (Bean, 1984; Sanguinetti and Kass, 1984). Bean described that for two states of the channel:

$$K_{app} = \frac{1}{1/K_L + (1 - L)/K_{inact}}$$

where the apparent dissociation constant (K_{app}) in a given experimental situation will depend on the high-affinity dissociation constant (K_{inact}) and the low-affinity dissociation constant (K_L) and the proportion of channels in the high-affinity state ($1 - L$). A further complication is that, if the high-affinity state is available for very short periods, there may not be time to allow equilibrium. These factors allow *apparent* tissue selectivity for DHPs whereby the drugs do not have time to block, for example, neuronal L-type channels activated by short action potentials, whereas L channels in smooth muscle, with membrane potentials of only -50 to -55 mV, are much more sensitive in that there is a greater predominance of the inactivated state at lower resting potentials. Functional experiments may be set up to explore the high-affinity component:



Thus, constant potassium depolarisation with 40 mM K^+ shifts the above equilibrium far to the right (approximately 70% inactivated channels in some tissues; Bean, 1984), allowing apparently competitive interactions with the channels, with high-affinity pharmacological effects of calcium antagonists with a similar order of potency to that seen in radioligand binding experiments (Spedding, 1982, 1985a,b; table 4). Changes in depolarisation will, therefore, be critical in the definition of drug potency. Other changes in the local environment such as acidosis have been claimed to make marked changes to the properties of calcium channels (Konnerth et al., 1987) which may change sensitivity to drugs, and the pH will modify drug potency when the pKa is in the appropriate range (Mannhold et al., 1984).

A further complication in functional and radioligand-binding studies reflects the highly lipophilic nature of many of the drugs used to investigate channel types. Uptake of DHPs and of other calcium antagonists into biological membranes may be very high with accumulation of 3,000-fold (nifedipine) to 19,000-fold (amlodipine) (Rhodes et al., 1985; Chester et al., 1987; Mason et al., 1991). This high concentration in the membrane may be in equilibrium with the receptor site so that the apparent

dissociation constant (K_{Dapp}) should also be modified by the membrane partition coefficient (K_{Pmem}) to give the local intramembrane dissociation constant, K_D , which may be up to four orders of magnitude different from the constant calculated from the concentration in the aqueous phase:

$$K_D = K_{Dapp} \cdot K_{Pmem}$$

The aqueous concentration may, therefore, be very different from the concentration close to the binding site in the channel.

Electrophysiological analysis must be used to assess channel subtypes and probable sites of action of drugs. Although electrophysiological techniques may be definitive for assessing whether drugs affect the appropriate ion currents, care must be taken in assessing the results, bearing in mind that very nonphysiological conditions may be used in the experimental protocol. Nevertheless, these techniques are definitive in designating the ionic selectivity of the channel under investigation and, hence, whether it may be classed as a calcium-selective channel in appropriate physiological conditions. Selectivity of drug action must be shown in electrophysiological experiments comparing effects on other channels in the same tissue. In these experiments, great care must be taken to assess the high-affinity component of a particular drug's action because the affinity for a particular channel state may be entirely dependent on the experimental conditions. Hence, experiments using a wide range of holding potentials, stimulation frequencies, charge carriers, etc. should be performed. Voltage or use dependency may markedly change affinity (see above).

2. *Radioligand-binding and autoradiographic studies.* Radioligand-binding and autoradiographic studies can indicate sites of action and channel distribution; these have been particularly useful in the definition of L-type channel distribution in which high-affinity ligands are readily available. The binding sites on the channels are similar to receptor sites, in that binding may be accompanied by marked changes in channel activity, but there may not be a known endogenous agonist for the site, which is one of the criteria for a "receptor." Stereospecific radiolabeled probes should be used to define the site of action on the channel subunit. Competitive interactions should be defined with other drugs sharing the same binding site. The preparation of a radiolabeled form of the drug in question that binds irreversibly to the channel (photoaffinity probes) is highly desirable in these experiments so that the labeled channel subunit may be purified and identified. Allosteric interactions with drugs that bind to other binding sites on the ion channel may be difficult to interpret because this type of interaction has been shown to be species, temperature, cation, tissue, and ligand dependent; in these cases, a wide variety of experimental procedures are required. Effects on modification of the dissociation rate of a radiolabeled ligand

by an unlabeled drug have been used to indicate whether a compound is acting at the same site as the ligand (unchanged dissociation rate) or at different, allosterically linked sites (faster or slower dissociation rate), but a wide range of concentrations of the drug should be used.

Binding experiments should be used to establish selectivity of action in that the affinity of new drugs for established sites on the Ca^{2+} channel should be compared with affinity for sites on other channels (e.g., [^3H]batrachotoxin binding on sodium channels).

3. Molecular biology. The primary structure of a channel may be deduced from the cDNA sequence and these sequences compared with those from other channels. Expression of the channel proteins from complementary RNA in frog oocytes or in mammalian cells allow for the identification and confirmation of the channel of interest. Further reconstitution experiments using purified proteins allow the best definition of channels, but allowance must be made for the influence of local biophase characteristics (second messengers, surface charge, membrane composition) on the performance of a channel in its natural environment. Antibodies raised against the channel subunits are useful in describing their role. The site of action of a drug can be assigned to a particular channel subunit when the appropriate radiolabeled probe is prepared. The definition of a site of action of a particular drug or toxin may be made by covalently binding the radiolabeled form to the channel and digesting and sequencing the labeled peptides.

It may be impossible to fulfill all the criteria for channel identification involving molecular biological techniques for which there are no high-affinity probes to label a channel; under these circumstances criteria 1 and 2 must be applied rigorously.

B. Classification of Binding Sites for Drugs on Calcium Channels

Clear criteria that must be fulfilled for classification of distinct binding sites for drugs on a particular channel are: (a) demonstration of a stereoselective binding site on the appropriate ion channel with competition from other agents sharing this site and appropriate interactions with other allosterically linked binding sites on the channel. Wherever possible, radiolabeled forms of the drug that can irreversibly bind to the channel (e.g., azido forms) should be synthesised so that the subunit to which the drug binds may be isolated and the amino acid sequence deduced; (b) demonstration of the appropriate electrophysiological changes in channel current following drug application, taking into account possible voltage- and use-dependent factors modifying drug action. Selectivity of action vis-à-vis other channels must be shown. The use of channel activators to reverse inhibitory effects is important; (c) functional studies should be compatible with the electrophysiological studies and clearly show

selectivity of action vis-à-vis other sites. Interactions with competing drugs and compounds should be defined. Reversal of inhibitory effects by channel activators may be a useful means of demonstration of the site of action; (d) selectivity windows should be constructed to indicate the site of action and relative affinities for T, N, L, P, Na^+ , K^+ , Cl^- channels and other receptor sites. It is important that the effects of any drug in an experimental system be considered, depending on its selectivity profile, so that effects are not erroneously ascribed to actions at a particular site.

IV. Types of Voltage-dependent Calcium Channels

The initial description of Ca^{2+} currents in the myocardium by Reuter (1967) initiated much work to define the properties of voltage-dependent Ca^{2+} channels, and there now appear to be many types of VDCCs. Tsien and colleagues (Nowycky et al., 1985; Tsien et al., 1986; Fox et al., 1987a,b; Tsien and Tsien, 1990) identified by electrophysiological and pharmacological means different channels which they called L type (for long lasting), T type (for transient, tiny), and N type (for neuronal, neither L nor T). This nomenclature is considered to be useful, but the potential multiplicity of channels means that future classifications may need to be revised. The channels are classified according to their activation and inactivation kinetics, their conductances, their ion specificity, and their sensitivity to drugs and toxins. Subsequently, Llinas and colleagues provided evidence that there may exist high threshold VDCCs in some neurones, and the channels were termed P-type channels (for Purkinje cells). An overview of the properties of the channels is shown in table 1, but it must be appreciated that channel properties vary among tissues and the channels may show different properties in other tissues. There may be a wealth of different types of VDCCs. Some electrophysiologists prefer to use "high- and low-threshold" channels, without further discrimination, but the existence of selective toxins and drugs points to major classes, which are best defined at present as in table 2.

A. Voltage-dependent Ca^{2+} -selective Channels

1. L-type channels. L-type channels, which are widely distributed in tissues, particularly in heart and smooth muscle, are highly sensitive to the DHPs, phenylalkylamines, and benzothiazepines and, consequently, are the channels in heart and smooth muscle targeted by Fleckenstein and Godfraind in the 1960s. For this reason, L-type channels may be considered as being DHP sensitive. There is a high density of L-type channels in skeletal muscle, and the availability of high-affinity radiolabeled probes allowed the primary structure of skeletal muscle L-type channels to be elucidated. L-type channels may consist of several subunits, known as α_1 , α_2 , β , γ , δ .

The α_1 subunit, from skeletal muscle, isolated on gels is about 165,000 Da and contains important phosphor-

TABLE 2
Classes of voltage-dependent calcium channels

Properties	Channel (conductance)			
	L (25 pS*)	N (12-20 pS)	T (8 pS)	P (10-12 pS)
Activation	High voltage	High voltage	Low voltage	Moderate high voltage
Inactivation	Slow	Moderate	Transient	Very slow
Location/function	Widespread, muscle and nerve	Neuronal transmitter release	Widespread pacemaker activity	Neuronal, Purkinje
Blockers	DHP, calciseptine, phenylalkylamines	Conotoxin	Flunarizine?	Funnel web spider toxin ϕ -aga-IVA

* 110 mM Ba²⁺ as charge carrier.

ylation sites and binding sites for some calcium antagonists. The protein has four repeating motifs (termed I to IV), each containing six putative membrane-spanning regions (termed S1 to S6; fig. 1), of which the S4 spans are the putative voltage sensor. Thus, the subunit strongly resembles sodium channels and is approximately 55% homologous to the sodium channel, especially in the membrane-spanning regions. Some drugs may, therefore, have affinity with both Na⁺ channels and L-type channels (Grima et al., 1986). The cytosolic regions are significantly divergent from the sodium channel and, hence, will be of great importance in identifying specific regions. The α_1 subunit has been cloned from rabbit skeletal muscle, heart, and lung, rat aorta, rat brain, and human heart. Northern analysis has revealed specific mRNA transcript sizes that are tissue specific (i.e., 8.5 kb in heart, 6.5 kb in skeletal muscle, 6.5 and 8.5 kb in aorta, and 6.5 kb in brain), which points to the probability of distinct isoforms of the L-type channel. (Insufficient studies have been reported to allow a clear determination of whether the L channel isoforms reflect differences among species or tissues, although the skeletal muscle isoform is clearly different.) A comparison of the deduced amino acids from the nucleotide sequences are consistent with different isoforms. The α_1 subunit [skeletal muscle structure: 1873 amino acids, 212 kDa (Tanabe et al., 1987; Ellis et al., 1988); rabbit heart (Mikami et al., 1989; Sligh et al., 1989); rat aorta and

brain (Koch et al., 1989); rabbit lung (Biel et al., 1990); fig. 1] apparently forms the ion-selective pore and voltage sensor and carries the binding sites for DHPs, phenylalkylamines such as verapamil, and benzothiazepines such as diltiazem. The cardiac subunit, but not the skeletal subunit, has been expressed in *Xenopus* oocytes and shown to have many of the properties of the L-type channel (Mikami et al., 1989).

Dysgenic mice (*mdg/mdg*), which have a lethal mutation (Beam et al., 1986) and an abnormal α_1 gene, no α_1 unit in the dystrophic skeletal muscle (Knudson et al., 1989) or L-type calcium current, and abnormal excitation-contraction coupling (Pincon-Raymond et al., 1985), have proven important experimental tools to define the critical role of the α_1 subunit. Tanabe et al. (1988) expressed the skeletal muscle α_1 form in myotubes from dysgenic mice and showed that expression normalised excitation-contraction coupling. This group went on (Tanabe et al., 1990a,b) to express the cardiac α_1 subunit in dysgenic myotubes and showed that this isoform of the α_1 subunit functioned as a "cardiac-type" voltage sensor and ion pore, even when expressed in skeletal muscle. Chimeric forms of the α_1 subunit were made to define which part of the molecule was critical for excitation-contraction coupling (Tanabe et al., 1990a,b). Perez-Reyes et al. (1989) expressed the α_1 subunit in murine L cells which have no other L channels and showed that DHP sites and Ca²⁺ current were ex-

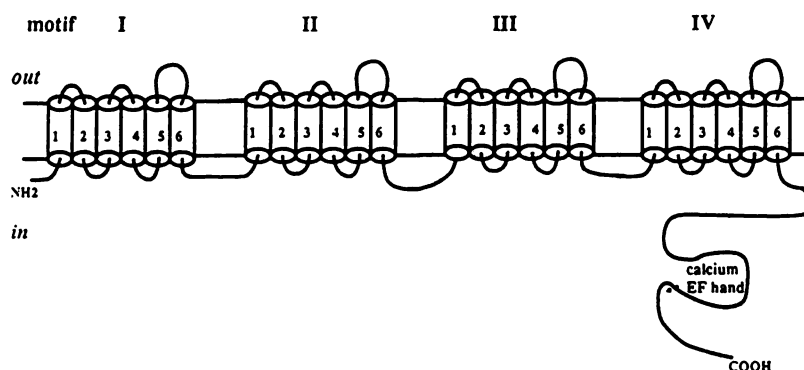


FIG. 1. Putative structure of α_1 subunit of L channel.

pressed, although abnormally. These experiments conclusively showed that the α_1 subunit is the critical voltage sensor and pore of the channel and apparently has the receptor sites for the drugs classed as calcium antagonists. The role of the other subunits has not been as well characterised as that of the α_1 subunit, and they appear to modulate the activity of the α_1 subunit (Singer et al., 1991). Furthermore, changes in the subunit composition or coupling may represent additional ways of increasing channel diversity.

An α_2 subunit (structure determined by Ellis et al., 1988), without binding sites for the drugs classed as calcium antagonists, does not act as a channel but with the δ subunit increases calcium current following injection of α_1 mRNA in *Xenopus* oocytes (Singer et al., 1991). The β subunit (structure determined by Ruth et al., 1989) has been shown to increase the rate of activation and inactivation of the channel and to modify DHP binding to the α_1 subunit (Varadi et al., 1991). The β subunit may markedly increase current when coexpressed with the α_1 unit (Singer et al., 1991), and a recent report (Varadi et al., 1991) indicates that the presence of the β unit increases the number of DHP-binding sites 12-fold and markedly increases kinetics; it remains to be explained how general this phenomenon is (Lacerda et al., 1991). A γ subunit (222 amino acids, structure determined by Jay et al., 1990) has been shown to occur in skeletal muscle; the function is unknown. A δ subunit may be linked by sulfhydryl bonds to the α_2 subunit.

There appear to be diverse forms of L-type channels, allowing tissue selectivity and diversity of function. The skeletal muscle α_1 subunit is smaller (212 kDa) than the cardiac form (242 kDa) and is encoded from a different gene; the skeletal muscle α_1 subunit may act as a voltage sensor for the sarcoplasmic reticulum calcium release channel which is ryanodine sensitive (Lai et al., 1988) and has several different properties from the cardiac subunit (McKenna et al., 1990). Both subunits resemble the sodium channel with approximately 55% homology in the membrane-spanning regions, which implies common ancestral forms. Rabbit heart, rat aorta and brain, and rabbit lung α_1 subunits have greater homology (>60%) and may arise from alternative splicing (Koch et al., 1989; Biel et al., 1990). Alternative splicing appears to be an important mechanism for producing numerous channel isoforms in mammalian tissues. Thus, voltage-dependent Ca^{2+} channels may be classed as in table 3, with N-, T-, P- and L-type channels and the L-type channels occurring in different isoforms.

L-type channels, particularly in the heart (Reuter, 1983; Brum et al., 1984), are also modulated by second-messenger systems. Several drugs may, therefore, indirectly modify channel function by acting at receptor proteins or second-messenger transducers. This committee takes the view that these indirect interactions with a wide variety of receptor and second-messenger proteins

TABLE 3
Classes of ion channel

1. Voltage-dependent Ca^{2+} -selective channels
L type (L1, 2, 3, 4 isoforms; α_1 , α_2 , β , γ , δ subunits identified)
T type
N type
P type
2. Other Ca^{2+} -selective channels
Ca^{2+} release channels in sarcoplasmic reticulum
Receptor-operated Ca^{2+} channels
3. Other voltage-dependent ion channels, without Ca^{2+} selectivity
Na^+ , K^+ , etc.

should be classed differently from direct binding of drugs to sites on ion channels (see below). Thus, although L-type channels may be modified by G proteins or adenylate cyclase, drugs modifying G proteins or adenylate cyclase should be classed as such and not as calcium antagonists.

In conclusion, voltage-dependent L-type Ca^{2+} channels are relatively well defined (table 4) because of the availability of high-affinity drugs to probe the channels and molecular genetic studies. L-type channels may be considered simply as those channels that are DHP sensitive.

2. *T-type channels.* T-type (for transient) channels have very different electrophysiological characteristics from L-type channels in that they require small depolarisations for activation and rapidly inactivate and may be important for pacemaker activity in several tissues. The T-type channel is located in a variety of tissues and has been found concentrated in the sinoatrial node (Bean, 1985), the atrioventricular node, specialized conducting tissue of the heart (Nilius et al., 1985; Tseng and Boyden, 1989), smooth muscle cells (Bean et al., 1986; Worley et al., 1986; Benham et al., 1987; Loirand et al., 1989; but see Bolton et al., 1988), and neurones (Carbone and Lux, 1984, 1987a,b; Nowycky et al., 1985), where they may be responsible for burst firing (White et al., 1989). There are few, or no, specific blockers of T-type channels (see below) and the lack of high-affinity probes has hindered structural definition.

3. *N-type channels.* The N-type channel is generally deemed to be sensitive to ω -conotoxins and in certain instances may be coupled to neurotransmitter release (Hirning et al., 1988; Miller, 1987), whereas selective antagonists of L-type channels do not normally modify transmitter release (Haeusler, 1972). Tsien and colleagues (Kongsamut et al., 1989) found that the presynaptic α_2 -adrenoceptor, when stimulated by noradrenaline, inhibits the movement of calcium through the N-type channel. Thus, some N-type channels, although predominantly voltage activated and hence contributing to neurotransmitter release or other events following depolarisation of nerve terminals, may be modulated by receptor-linked second-messenger systems, such as G proteins. There may be a wide range of N channels in that the electrophysiological characteristics of N channels vary among different tissues, but these channels are

TABLE 4
Summary of L channel characteristics

Characteristic	Description
Endogenous ligand	None identified so far
Coupling class	Ion channel
Type	VDCC
Subtype	L channel
Previous name	Slow channel
Functional assays (response)	Inhibition and stimulation of calcium current and of excitation-response coupling
Selective activators	DHP (Bay K 8644, CGP 28392, (+)-202-791)
Selective inhibitors	DHPs, benzothiazepines, phenylalkylamines (table 3)
Potencies	See text and table 6
Radioligand-binding assays	Membrane preparations from heart, brain, smooth muscle, skeletal muscle, cell lines
Radioligands	DHPs: [³ H]Nitrendipine, [³ H]nimodipine, [³ H]isradipine, [³ H]Bay K 8644, iodipine; benzothiazepines: [³ H]diltiazem, [³ H]clentiazem; phenylalkylamines: [³ H]verapamil, [³ H]devapamil; Others: [³ H]SR33557, [³ H]HOE 166, [³ H]fluspirilene
Ligand affinities	DHPs, <1 nM; benzothiazepines, 3–500 nM; phenylalkylamines, 2–50 nM; see table 6 and text
Binding site distribution	Widespread in excitable tissues
Transducing mechanisms	Voltage-dependent ion channel, may be modified by G proteins, calmodulin, protein kinase C and cyclic AMP-dependent protein kinases
Structural information	See text and figure 1

also coupled to a variety of second-messenger systems modifying transmitter release (e.g., G proteins) which may alter function. ω -Conotoxin binds to and inhibits N-type channel activity, blocking transmitter release. A range of conotoxins, such as SNX-111 from *Conus magus* and SNX-183 from *Conus striatus*, may have selectivity for N channel subtypes (Gohil et al., 1991).

4. *P-type channels*. P-type channels were proposed by Llinas et al. (1989a,b) on the basis that DHP- and conotoxin-resistant currents in cerebellar Purkinje and granule cells and in squid giant axons were susceptible to funnel web spider venom; in squid giant axon, transmitter release was dependent on this channel and ω -agatoxin IVA may be the most selective toxin (Adams et al., 1992). These channels may form a larger proportion of calcium channels in the brain than was hitherto supposed (Leonard et al., 1987; Lin et al., 1990; Regan et al., 1991) and may be responsible for neurotransmitter release in cell types in many brain areas (Hillman et al., 1991). The channels show little inactivation. Recently, Mori et al. (1991) defined, from brain cDNA, the primary structure of a calcium channel (BI protein) with similar pharmacological characteristics to the P channel; channel activity was expressed in *Xenopus* oocytes and was dramatically increased by the coexpression of the α_2 and β subunits.

Therefore, conclusive evidence exists for a range of calcium channels from experiments using molecular biological, electrophysiological, and radioligand-binding techniques. At present, we consider that the main channel classes can best be accommodated in an "L, T, N, P" classification. However, Snutch et al. (1990) identified four distinct classes of cDNAs from rat brain (A, B, C, D) with the class C polypeptide being 97% homologous to the heart α_1 subunit. The putative voltage-sensor unit (S4) differed between A,B and C,D, which implies

potential differences in electrophysiological properties. Snutch et al. (1990) estimated that there are a minimum of eight different calcium channel transcripts in rat brain. It is likely that there exist many more types of VDCCs. It is also likely that small changes in amino acid sequence will lead to marked changes in electrophysiological properties, ion selectivities, and sensitivity to drugs. The classification described here is useful in that marked functional differences in electrophysiological properties and drug sensitivity can be accommodated.

B. Other Ca^{2+} -selective Channels

Many Ca^{2+} channels may not be primarily voltage dependent but regulated by other factors. The Ca^{2+} channel in the sarcoplasmic reticulum is a prime determinant of contractility in many muscle types and is tightly linked with L-type channels in skeletal muscle; ryanodine is the most selective agent for this channel. Selective Ca^{2+} channels that refill intracellular calcium stores have been described in mast cells (Hoth and Penner, 1992); these channels may be highly Ca^{2+} selective in that they are not permeable to Mn^{2+} . Furthermore, some Ca^{2+} channels in the cell membrane may be activated by intracellular Ca^{2+} release and by inositol 1,3,4,5-tetrakisphosphate (e.g., in endothelium, Luckhoff and Clapham, 1992), linking receptor activation to Ca^{2+} entry.

Receptor systems may be directly linked to channels with some selectivity for Ca^{2+} under certain conditions. In these systems receptor activation rather than voltage-dependent activation is the prime trigger, although there may be some modulation by voltage. Thus, Bolton (1979) hypothesised the existence of receptor-operated Ca^{2+} channels, and data (Godfraind et al., 1986; Haeusler and De Peyer, 1989; Hallam and Rink, 1989) support the existence of such channels, although selectivity for calcium may be low. In the rabbit ear artery, α -adrenoceptor

activation opens voltage-independent cation channels that are not selective for calcium (Amedee et al., 1989). Unfortunately, at present, the molecular biology of these channels is not at the same stage as with voltage-dependent channels [and it remains to be seen to what extent the channel activation mechanisms described by Luckhoff and Clapham (1992) explain some of the receptor-mediated channel activity], but these will be listed as primary targets for drug action: ionotropic excitatory amino acid receptor-linked channels, noradrenaline-linked channels in smooth muscle, and nucleotide/nucleoside-linked channels (Benham and Tsien, 1987).

Of the ionotropic excitatory amino acid receptor-linked channels that respond to glutamate, the N-methyl-D-aspartate-gated channels (Moriyoshi et al., 1991) have been shown, rather nonselectively, to conduct Ca^{2+} , whereas kainate- and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate-gated channels do not (but see Hollmann et al., 1991). A new class of Ca^{2+} -permeable channels that respond to glutamate but are not N-methyl-D-aspartate channels have been recently reported (Gilbertson et al., 1991).

C. Other Voltage-dependent Ion Channels

The many other voltage-dependent ion channels that are not selective for Ca^{2+} represent potential targets for drugs, particularly if there is homology between receptor sites on these channels and on Ca^{2+} channels (e.g., the Na^+ channel, see above). Selectivity for calcium channels must be assessed by comparing affinity with other channels. Because some drugs may be highly use or voltage dependent, these factors must be taken into account, and comparisons of affinity should be made in the same tissues if possible.

D. Nonchannel Targets

Drugs may modulate many aspects of Ca^{2+} mobilisation without affecting ion channels, and some of these aspects are: Ca^{2+} ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchange, functioning of the sarcoplasmic reticulum, mitochondrial Ca^{2+} transport and the coupling of intracellular Ca^{2+} levels with oxidative metabolism, sensitivity of the contractile proteins, cell nuclei.

V. Drug-binding Sites on Calcium Channel Proteins

The classes of binding sites as defined by the most selective drugs are shown in table 5.

A. Class 1: L Channel-selective Agents

The drugs classed as calcium antagonists which were used in the early studies by Fleckenstein's and Godfraind's groups have been found subsequently to inhibit Ca^{2+} entry into cells via L channels.

a. Dihydropyridines. Criteria for distinct binding sites for DHPs on the α_1 subunit of the L channel are fulfilled, and many DHPs have very high affinity for the L-type

TABLE 5
Drug-binding sites on calcium channel proteins

- | |
|---|
| 1. Agents interacting selectively with binding sites on L-type channels |
| a. DHP site (nifedipine, nicardipine, nitrendipine, nisoldipine, felodipine, isradipine, darodipine, flordipine, amlodipine, nimodipine, niguldipine, niludipine, oxodipine, riodipine, lacidipine, elgodipine) |
| b. Benzothiazepine site (diltiazem, clentiazem, diclofurime) |
| c. Phenylalkylamine site (verapamil, gallopamil, levemopamil, anipamil, devapamil, tiapamil) |
| 2. Compounds acting at other undefined sites on L channels (SR 33557, HOE 166, McN6186, MDL12330A, MCI176, pinaverium, fluspirilene) |
| 3. Agents interacting selectively with other voltage-dependent Ca^{2+} -selective channels (no agent is known to be highly selective at these sites) |
| a. T channels (flunarizine? tetrandine? see text) |
| b. N channels (conotoxins; see text) |
| c. P channels (funnel web spider toxins? see text) |
| 4. Nonselective channel modulators (fendiline, prenylamine, bepridil, caroverine, cinnarizine, flunarizine; see text) |
| 5. Agents acting at other Ca^{2+} -selective channels |
| a. Ca^{2+} release channels in sarcoplasmic reticulum (ryanodine, not an antagonist) |
| b. Receptor-gated channels |
| i. Excitatory amino acid channels |
| ii. α -Adrenoceptor-linked channels |
| iii. Angiotensin-linked channels |
| iv. Nucleotide/nucleoside-linked channels |

channel. Although some affinity for other sites (mitochondrial, calmodulin, nucleoside transporter, etc.) has been demonstrated, the stereoselectivity shown at these sites is either absent or not similar to that shown for the highly stereoselective interaction with the L-type channel. Some of the DHPs may be protonated at physiological pH (nicardipine, amlodipine, niguldipine) which may lead to different cellular distribution or kinetics at the channel. DHP calcium antagonists such as nifedipine have been well characterised to be highly potent inhibitors of L-type channel function with use-dependent effects and low nanomolar affinity for the inactivated state of the channel but much less affinity for other states (e.g., closed, open) (Bean, 1984, Sanguinetti and Kass, 1984). The availability of ^3H -DHPs as probes (Belleman et al., 1981; Glossmann et al., 1985; Glossmann and Ferry, 1983; Janis et al., 1987) allowed the demonstration of high-affinity binding that was Ca^{2+} dependent, although only very low concentrations ($>1 \mu\text{M}$ Ca^{2+} , i.e., concentrations normally present in trace amounts in buffer) were required to allow full demonstration of binding. Receptor distribution in the brain has been mapped (Murphy et al., 1982; Cortes et al., 1983, 1984). DHP calcium channel activators such as Bay K 8644 (Schramm et al., 1983) displace antagonist binding but increase Ca^{2+} current (Hess et al., 1984; Brown et al., 1984; Sanguinetti et al., 1986) and have selective func-

tional interactions with other classes of channel modulators (Su et al., 1984; Spedding and Berg, 1984).

Definition of the binding site of DHPs on the α_1 subunit has been claimed (Striessnig et al., but also see Regulla et al., 1991), but further confirmation of the amino acids involved in the binding site is required because the claims are conflicting. DHP binding to L-type channels has an absolute requirement for divalent cations ($>1 \mu\text{M}$), and the amino acid sequence with the greatest proportion of DHP binding in the study of Regulla et al. (1991) abuts the putative cytosolic calcium-binding domain in the channel (EF hand, Babitch, 1990). In contrast, the site described by Striessnig et al. (1991) is accessible from the extracellular surface.

The equilibrium dissociation constants (K_d) and receptor densities (B_{max}) may vary with the DHP ligand used, the temperature, the tissue, and the pH (maximal specific binding over the pH range 6.5 to 7.7) (Glossmann et al., 1985; Janis et al., 1987; Kenny et al., 1991). Affinities of DHPs for L channels in functional and binding experiments have been listed in comprehensive reviews (Godfraind et al., 1986; Janis et al., 1987) and affinities from one laboratory are listed in table 6. The values in table

6 represent affinity for one isoform of the channel (smooth muscle)

b. Benzothiazepines. Benzothiazepines such as diltiazem have a distinct binding site on the α_1 subunit, and this site is linked allosterically to the DHP site. Interactions with this site and the DHP site are highly ligand and temperature dependent (Glossmann et al., 1985; Galizzi et al., 1986; Mir and Spedding, 1987); the azido ligand of diltiazem has been used to covalently label the α_1 subunit of the L-type channel (Vaghy et al., 1987; Naito et al., 1989)

c. Verapamil-like agents. The site of action of verapamil-like agents is distinct from the DHP and benzothiazepine sites but linked to the 1a and 1b sites; a number of compounds have high affinity for this site (Table 3). The distribution of brain L-type channels labeled with (-)-[^3H]desmethoxyverapamil is identical with distribution of channels with affinity for diltiazem or DHPs (Ferry et al., 1984). Phenylalkylamine verapamil analogues such as [^3H]LU49888 label amino acids 1350 to 1390 in skeletal muscle, i.e., a different but closely opposed site to the DHP site (Striessnig and Catterall, 1991).

TABLE 6

Binding and functional data of (+)isradipine, (-)isradipine, nisoldipine, (-)nimodipine, (+)nimodipine, nifedipine, and flunarizine in rat vessels*

Drug (preparation)	Binding studies (K_D or K_i , nM)			Functional studies (IC_{50} , nM)	
	Membranes	Intact tissue		K^+ contraction (at 30–35 min)	K^+ - $^{45}\text{Ca}^{2+}$ influx (at 33–35 min)
		Physiological medium	K^+ (100 mM)		
(+)Isradipine					
Aorta	0.085 ^a		0.068 ^b	0.092 ^a	
Mesenteric artery	0.055 ^c	0.2 ^d	0.044 ^d	0.033 ^d	
Cerebral microvessels		0.113 ^c	0.024 ^c		
(-)Isradipine					
Aorta	14.9 ^a			12.3 ^c	
Mesenteric artery		23 ^d	1 ^d	5 ^d	
Cerebral microvessels		10.1 ^c	5.9 ^c		
Nisoldipine					
Aorta	0.148 ^a		0.067 ^b	0.071 ^a	0.042 ^b
Mesenteric artery		0.263 ^b	0.058 ^b	0.049 ^b	
Cerebral microvessels					
(-)Nimodipine					
Aorta	0.546 ^f			0.234 ^f	
Mesenteric artery					
Cerebral microvessels		0.77 ^c	0.23 ^c	0.14 [†]	0.1 [†]
(+)Nimodipine					
Aorta	2.89 ^f			1.371 ^f	
Mesenteric artery					
Cerebral microvessels		5.9 ^c	2.0 ^c	0.14 [†]	0.1 [†]
Nifedipine					
Aorta	3.59 ^a			1.3 ^a	1.6 ^a
Mesenteric artery	3 ^c	3.1 ^c	1.2 ^c	1.9 ^a	1.9 ^a
Cerebral microvessels		2.8 ^c	2.9 ^c		2 ^d
Flunarizine					
Aorta				19 ^g	37 ^g
Mesenteric artery	500 ^c	>>1000 ^c	900 ^c	2 ^c	
Cerebral microvessels				8 ^f	4 ^f

* References: ^a Wibo et al., 1988; ^b Morel and Godfraind, 1991; ^c Morel and Godfraind, 1988; ^d Morel and Godfraind, 1987; ^e Morel and Godfraind, 1989; ^f Godfraind et al., 1985; ^g Morel et al., 1990; ^h Godfraind, 1983; ⁱ Godfraind and Dieu, 1981.

† Racemic mixture.

Recently, several compounds (SR33557: Schmid et al., 1989; Polster et al., 1990; HOE 166: Striessnig et al., 1988; pinaverium: Beech et al., 1990; fluspirilene: Qar et al., 1987; King et al., 1989; Kenny et al., 1990) have been shown to have affinity for L-type channels with potentially novel sites of action. However, at present, there is insufficient evidence according to our criteria to allow assignation of them to classes 1a, 1b, or 1c or to allow designation of a new class. A new class may be designated when all the criteria have been fully met. Nevertheless, it is to be anticipated that several new sites will be defined in the near future.

B. Class 2: Agents Interacting with Other Voltage-dependent Ca^{2+} Channels

There are no highly selective blockers of T or N channels, although Ni^{2+} has some selectivity for T channels compared with Cd^{2+} , whereas Cd^{2+} is more potent at L and N channels. Several agents may block T channels, and there may be some differences in potency depending on the tissue (e.g., rat hypothalamic neurones: flunarizine > nicardipine > nifedipine > nimodipine > diltiazem; Akaike et al., 1989; see also Tytgat et al., 1988; Van Skiver et al., 1988). However, the selectivity of the effects of flunarizine have still to be defined vis-à-vis sodium channels (Grima et al., 1986; Pauwels et al., 1991), P-type and N-type (Tytgat et al., 1991) channels, and therefore, agents such as flunarizine cannot be definitively assigned to this class yet. Ethosuximide may have selectivity for T-type channels compared with sodium channels, and tetrandine has recently been shown to have some selectivity for T channels compared with L channels in neuroblastoma cells (Liu et al., 1991). Some DHPs (felodipine, Van Skiver et al., 1988; nicardipine, Akaike et al., 1989) may also block T channels, but these drugs are still selective for L channels and are, therefore, grouped as class 1a agents. ω -Conotoxin has selectivity for N channels. Funnel web spider toxins (Llinas et al., 1989a,b) were used to designate P channels, but there are many different and poorly quantified toxins in most extracts; one purified form, ω -agatoxin-IVA (Mintz et al., 1992), has been used to define P channels.

C. Class 3: Nonselective Channel Modulators

Several drugs that are important in a variety of pathological conditions clearly interact with L channels but in a manner distinct from class 1 agents, as assessed by functional and radioligand-binding tests and interactions with L channel activators (e.g., flunarizine, etc., Spedding, 1985a,b). These drugs are, therefore, calcium antagonists and will selectively reverse the effects of DHP calcium channel activators such as Bay K 8644 (Spedding and Berg, 1984). However, these agents have low selectivity for L channels in electrophysiological and radioligand-binding studies, indicating either that the drugs have genuinely low selectivity or that the molecular means of analysing their interactions with L channels

are nonoptimal. Because the functional profiles of this class of agents indicate differences from class 1 agents and there is no selectivity for L channels, these drugs are currently classed separately.

VI. Nomenclature

The terms "calcium antagonist" or "calcium entry blocker" have gained historic pharmacological and clinical acceptance when applied to agents inhibiting L-type channel function by acting at sites 1a, 1b, or 1c, and these terms will undoubtedly continue to be used to describe the classic pharmacological properties of this type of agent. However, it is clear that new pharmacological profiles may be expected from interactions with other types of calcium channel, or even from new binding sites on the L-type channel, which will be quite different from the profile associated with, for example, nifedipine. Thus, the term "calcium channel modulator" is preferred for agents interacting with calcium channels. The term "calcium agonist" is inappropriate, and agents such as Bay K 8644 should be referred to as calcium channel activators and inhibitory compounds should be referred to as calcium channel blockers.

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