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Topical Review

Calcium Channels: Molecular Pharmacology, Structure and Regulation

M. Marlene Hosey† and Michel Lazdunski‡
†Department of Biological Chemistry and Structure, University of Health Sciences, The Chicago Medical School, North Chicago, Illinois 60064, and ‡Centre de Biochimie du CNRS, Université de Nice, 06034 Nice, France

Introduction

It is well recognized that calcium (Ca) is an important regulatory element for many cellular processes. In most eukaryotic cells a diverse array of Ca transporting systems functions to maintain the steep concentration gradient between extracellular Ca, estimated to be in the millimolar range, and intracellular Ca, which can vary between 0.1–10 μM, depending on the state of the cell. Specific and distinct pathways exist by which Ca enters and exits cells. Several different types of Ca transport systems serve to maintain the low concentration of intracellular Ca, by transporting Ca either out of the cell or into intracellular storage sites (for review, *see* Carafoli, 1987). These *exit* pathways have been extensively studied, and until recently our knowledge of these systems was far greater than for pathways involved in Ca *entry*. The major entry pathway for Ca in many cell types is via plasma membrane Ca channels. Considerable progress has now been made in elucidating the properties of certain Ca channels. The purpose of this review is to summarize our current understanding of the molecular properties of Ca channels; particular emphasis will be given to the pharmacological and biochemical characterization of Ca channels, as well as the mechanisms by which they are regulated by neurotransmitter-mediated processes.

Functions and Types of Ca Channels

Ca channels can be thought of as structures that form functional pores in the plasma membrane. Ca channels are normally closed; when opened, Ca passively flows through the channels along the Ca electrochemical gradient. Several million Ca ions/sec can enter cells through open Ca channels. Ca channels are important in supplying Ca to many types of cells, particularly to excitatory cells such as muscle and nerve, where they are postulated to play a role in excitation-contraction coupling and neurotransmitter release, respectively (for reviews, *see* Reuter, 1983; Tsien, 1983; Avila-Sakar et al., 1986; McCleskey et al., 1986; Miller, 1987*a*; Morad & Cleemann, 1987). Ca channels are also present in secretory cells (Baker, Knight & Knight, 1981; Fenwick, Marty & Neher, 1982; Luini et al., 1986) and other nonexcitable cells such as lymphocytes (Fukushima & Hagiwara, 1983), sperm (Kazazoglou et al., 1985) and neutrophils (von Tscharner et al., 1986), and probably even in plant cells (Graziana et al., 1988) where regulation of Ca entry is also important for cell function. Ca channels are normally present in plasma membranes, but some forms of Ca channels appear to be present in intracellular membranes as well (Fabiato, 1983; Fleischer et al., 1985; Smith, Coronado & Meissner, 1986; Suarez-Isla et al., 1986; Imagawa et al., 1987*b*; Palade, 1987), where they have an important function in the process of the release of Ca from intracellular storage sites.

Different types of Ca channels are known to exist and are characterized by fundamental differences in the mechanisms governing their opening and closing (Fig. 1). Some Ca channels are voltage dependent and open in response to an appropriate membrane depolarization. Within this category there are subclasses of Ca channels that differ in their voltage sensitivities, kinetic properties, pharmacological sensitivities, etc. (Carbone & Lux, 1984; Bean, 1985; Bossu, Feltz, & Thomann, 1985; Fedulova, Kostyuk, & Veselovsky, 1985; No-

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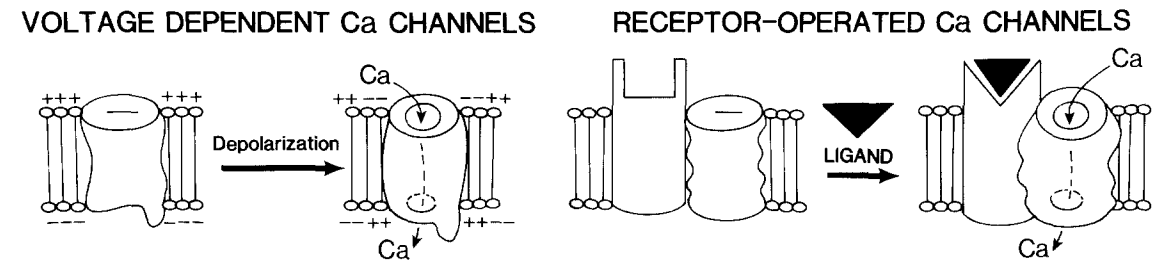

	T TYPE	N TYPE	L TYPE	EXAMPLES	RECEPTOR	CELL
Activation	> -70 mv	> -30 mv	> -10 mv		ATP, Vasopressin	Smooth muscle
Conductance	Tiny	Moderate	Large		Mitogens	Lymphocytes
	Transient	Transient	Long-lasting		Parathyroid hormone	Osteosarcoma
Sensitive to					f-Met-Leu-Phe	Neutrophils
DHPs	No	No	Yes		NMDA	Neurons
ω -CT	No	Yes	Yes			

Fig. 1. Hypothetical models of the different types of Ca channels that are controlled by voltage or receptor-dependent mechanisms. *See* text for details

wycky, Fox & Tsien, 1985*a*; Cognard, Lazdunski & Romey, 1986*a*; Fox, Nowycky & Tsien, 1987*a,b*). Three types of voltage-dependent Ca channels (Fig. 1) have been extensively studied (reviewed in McCleskey et al., 1986, and *see* Fox et al., 1987*a,b*). These are referred to as: (i) the L-type (Nowycky et al., 1985*a*; Fox et al., 1987*a,b*), high-voltage activated (Carbone & Lux, 1984) or slow (Fedulova et al., 1985; Cognard et al., 1986*a*) channel, which conducts a long-lasting current of large conductance (~25 pS); (ii) the T-type (Nowycky et al., 1985*a*; Fox et al., 1987*a,b*), low-voltage activated (Carbone & Lux, 1984) or fast (Fedulova et al., 1985; Cota & Stefani, 1986; Cognard et al., 1986*a*) channel, which is activated at low voltages and is characterized by transient currents with small conductance (~9 pS); and (iii) the N-type channel (Nowycky et al., 1985*a*; Fox et al., 1987*a,b*) which is neither T nor L, but is activated at relatively high voltages and conducts a relatively transient current of intermediate size (~15 pS). One or more types of voltage-dependent Ca channels may exist in a particular cell type. For example, in sensory neurons of dorsal root ganglia, T, N and L-type Ca channels co-exist (Nowycky et al., 1985*a*; Fox et al., 1987*a,b*; Gross & Macdonald, 1987). In sympathetic neurons of superior cervical ganglia, L and N, but not T, channels exist (Wanke et al., 1987). In cardiac and skeletal muscle, both L and T type, but not N, channels have been observed (Bean, 1985; Nilius et al., 1985; Mitra & Morad, 1986; Cota & Stefani, 1986; Cognard et al., 1986*a*). Similarly, in smooth muscle, L and T type Ca channels have been characterized (Friedman et al., 1986; Loirand et al., 1986; Sturek & Hermsmeyer, 1986; Benham, Hess & Tsien, 1987; Yatani et al., 1987*a*). It appears that there also may be subtypes of the different varieties of voltage-dependent Ca channels, since, for example, the L-type Ca channel present in the heart differs in certain pharmacological and electrophysiological properties from the L-type Ca channel present in skeletal muscle (Glossman et al., 1984; Cognard et al., 1986*a*; Rosenberg et al., 1986; Fosset & Lazdunski, 1987), and in other aspects from L type channels in neurons (McCleskey, et al., 1987). The different types of voltage-dependent Ca channels may serve different functions (*see* Perney et al., 1986; Miller, 1987*a*); however, more information is required to establish the precise role of each type of Ca channel in different cell types.

The second major category of Ca channels includes channels that are operated through receptor-dependent mechanisms (Fig. 1). These channels are often referred to as receptor-operated channels (Bolton, 1979; van Breemen, Aaronson & Loutzenhiser, 1979), and are opened in response to activation of an associated receptor. Typical channels of this type include the nonspecific ion channel associated with the nicotinic acetylcholine receptor (for review, *see* Changeux, Devillers-Thiery & Chemouilli, 1984) and chloride channels associated with GABA and glycine receptors (Iversen, 1984). Some receptor-associated ion channels are permeable to Ca and could therefore be considered as receptor-operated Ca channels. Examples include channels in smooth muscle that are opened by activation of ATP receptors (Benham & Tsien, 1987) and neuronal ion channels that are associated with a particular subtype of glutamate receptors referred to as N-methyl-D-asparate (NMDA) receptors (Ascher & Nowak, 1986). Although much less is known about receptor-operated Ca channels than voltage-depen-

dent Ca channels, they are currently the subject of extensive investigation. Several recent reports strongly suggest that, as is the case for voltage-dependent Ca channels, different types of receptor-dependent Ca channels may exist. For example, a mitogen-activated Ca channel in T lymphocytes appears to be opened by the second messenger inositol 1,4,5-triphosphate (IP_3) (Kuno & Gardner, 1987), whereas a nonselective ion channel which passes Ca in response to formyl peptide activation of neutrophils appears to be activated by a rise in intracellular Ca, but is insensitive to IP_3 (von Tscharner et al., 1987). Other types of receptor-dependent Ca channels have also been described including vasopressin-activated Ca channels in smooth muscle cells (Wallnofer, Cauvin & Ruegg, 1987; Zschauer et al., 1987) and parathyroid hormone activated Ca channels in osteosarcoma cells (Yamaguchi et al., 1987*a*). While it is still too early to classify the potentially different species of receptor-dependent channels, it seems certain that we will learn more about these types of Ca channels in the near future.

In addition to Ca channels that are strictly receptor-operated channels, an interesting feature of some Ca channels is that they are *regulated* by receptor-dependent processes. Thus, in addition to the fundamental mechanisms governing their opening and closing, a growing number of Ca channels, including voltage-dependent Ca channels, are subject to regulation by more than one mechanism. Certain Ca channels are regulated by receptor-activated GTP-binding proteins (G proteins) (Dunlap, Holz & Rane, 1987). In addition, some Ca channels are regulated by a variety of reversible phosphorylation and dephosphorylation events which occur in response to receptor-dependent alterations in intracellular second messengers (Reuter, 1983; Tsien et al., 1986; Miller, 1987*a*). Thus, while these channels need not be strictly "receptor-operated" channels, they are subject to regulation by receptor-dependent processes. These regulatory mechanisms will be discussed in more detail in the final section of this review.

Ca Channels Have an Extensive and Complex Pharmacology

Insights into the properties and functions of Ca channels were first obtained from electrophysiological studies (reviewed in Reuter, 1984; Tsien, 1983). The recent development of the patch-clamp technique (Hamill et al., 1981; reviewed in Sakmann & Neher, 1984) and related technology have made it possible to analyze the properties of ion channels at the single channel level. As a result, our understanding of the molecular properties of Ca channels, from a biophysical viewpoint, has increased at a phenomenal rate (*see* Tsien, 1983; Reuter, 1984; McCleskey et al., 1986; Reuter, Kokubun & Prod'hom, 1986; Miller, 1987*a*). Complementary to the electrophysiological studies have been extensive pharmacological and biochemical investigations (reviewed in Schwartz & Triggle, 1984; Godfraind, Miller & Wibo, 1986; Triggle & Janis, 1987; Fosset & Lazdunski, 1987; Miller, 1987*b*; Scriabine, 1987) which were sparked by the discovery of a fairly large variety of agents that have selective effects on Ca channels. Not only are some of these agents clinically useful in the treatment of cardiovascular disorders, including angina and hypertension (*see* Janis & Triggle, 1984), but they have been, and continue to be, useful as biochemical probes to elucidate the molecular properties of Ca channels.

Drugs that specifically interact with Ca channels were initially developed because of their potential usefulness in cardiovascular disorders (*see* Fleckenstein, 1983). The drugs that have received the most widespread attention belong to three distinct chemical classes: (i) the 1,4-dihydopyridine (DHP) derivatives such as nitrendipine, nimodipine, PN 200-110, and Bay k 8644; (ii) the phenylalkylamines such as verapamil, D-600, and D-888; and (iii) the benzothiazepines, typified by diltiazem (*see* Fig. 2 for structures). In addition, there are many other drugs which have effects on Ca channels; among those to be mentioned herein are bepridil and the diphenylbutylpiperidines (Fig. 2). Others include those of the flunarizine/cinnarizine series, which will not be discussed here, but have been extensively reviewed by Godfraind et al. (1986). All of the drugs initially developed *inhibited* Ca channels and were collectively referred to as "Ca channel antagonists," "Ca channel blockers," or "Ca channel inhibitors." Drugs capable of *activating* Ca channels, such as Bay k 8644 and CGP 28392 (Schramm et al., 1983; Brown, Kunze & Yatani, 1984; Freedman & Miller, 1984; Hess, Lansman & Tsien, 1984; Kokubun & Reuter, 1984), have now been developed and have been referred to as "Ca channel agonists" or "Ca channel activators." In view of the multiplicity of actions that drugs could have to achieve inhibition or activation of Ca channels, the more general term "Ca channel effectors" will be used in this review as a collective term to describe these drugs.

Ca channel effectors have been the subjects of extensive reviews (Janis & Scriabine, 1983; Glossmann et al., 1984; Schwartz & Triggle, 1984; Godfraind et al., 1986; Scriabine, 1987; Miller, 1987*b*; Fosset & Lazdunski, 1987; Triggle & Janis, 1987)

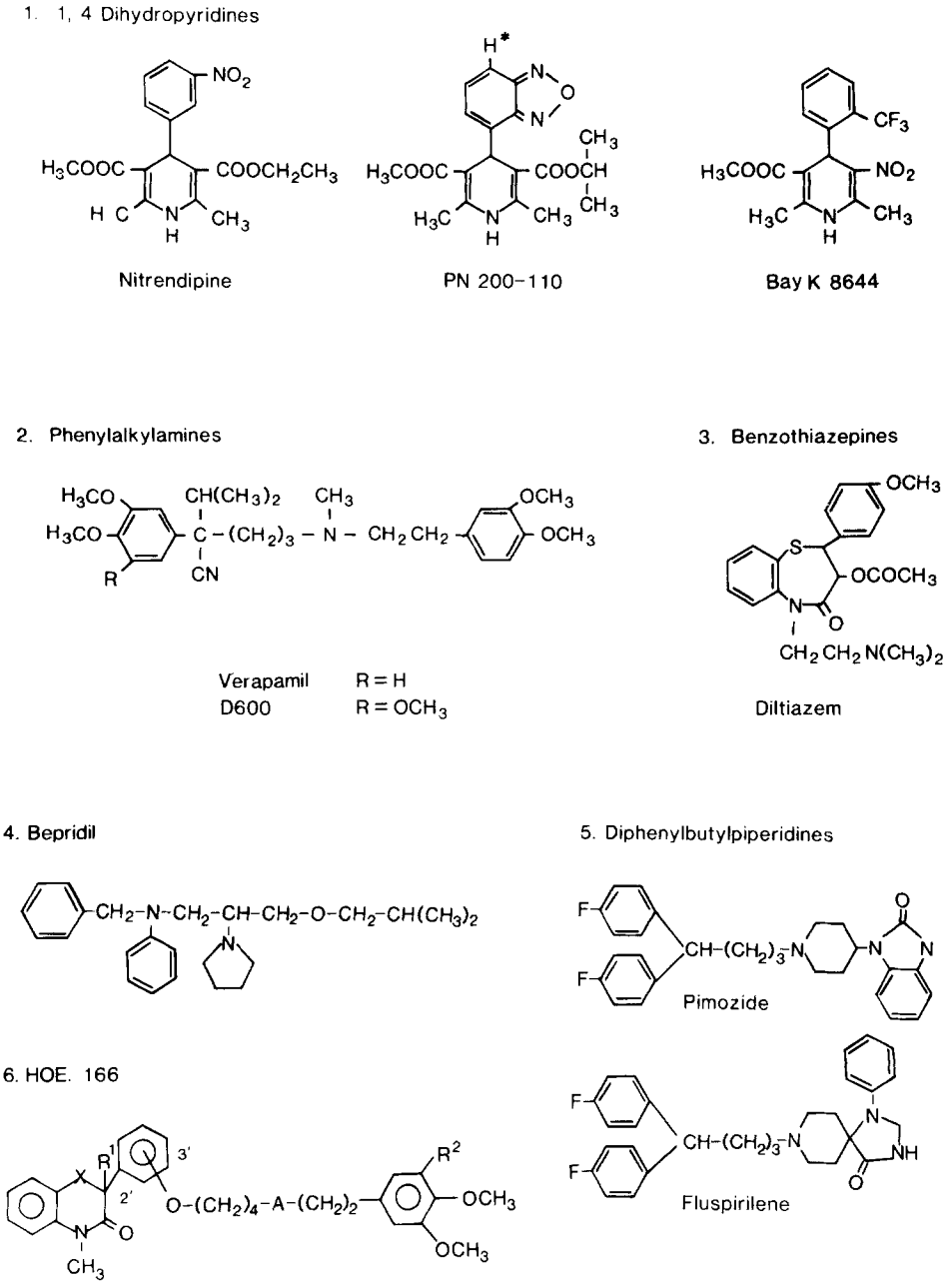

Fig. 2. Structures of some drugs which bind to receptors associated with Ca channels. *See* text for details

and only several features will be briefly touched on here. Most of the drugs developed to date appear to have selective effects on the L-type or slow voltage-dependent Ca channel. However, it should be noted that most studies carried out so far have not systematically tested all types of drugs on the different types of Ca channels. What is clear at present pertains mostly to the dihydropyridines in that these drugs appear to be without effect on other types of voltage-dependent Ca channels, including the T-type and N-type (Nowycky et al., 1985*a*; Friedman et al., 1986; Loirand et al., 1986; Fox et al., 1987a,b; McCleskey et al., 1987; Yatani et al., 1987*a*), as well as receptor-operated Ca channels (*see* for example Wallnofer, et al., 1987 and Zschauer et al., 1987). Because the L-type channel is the only Ca channel identified to date that is sensitive to the 1,4-dihydropyridines, this channel is also often referred to as the dihydropyridine-sensitive Ca channel. Electrophysiological studies have clearly shown that the L-type Ca channel can exhibit three different models of gating behavior, and that activators

and inhibitors of the DHP series can dramatically influence which mode of channel gating is expressed (Hess et al., 1984; Kokubun & Reuter, 1984; Nowycky, Fox & Tsien, 1985*b*).

Multiple Drug Receptors are Present on L-Type Ca Channels and Are Modulated by Many Factors

The availability of Ca channel effectors in radiolabeled forms has led to the demonstration that L-type Ca channels possess several specific high-affinity receptors for these agents. One receptor specifically binds, 1,4-dihydropyridine derivatives with dissociation constants in the pM-nM range (Glossmann et al., 1982; Bolger et al., 1983; Ferry, Goll & Glossmann, 1983; Fosset et al., 1983; Janis et al., 1984*b*; Janis & Triggle, 1984). Present evidence suggests that a single dihydropyridine receptor is responsible for binding both Ca channel activating and inhibiting dihydropyridines (Janis et al., 1984*a*; Williams et al., 1985; Kokubun et al., 1986; Hamilton et al., 1987; Maan & Hosey, 1987). However, some recent data (Kokubun et al., 1986; Brown, Kunze & Yatani, 1986) suggests that there may be more than one high affinity binding site associated with the dihydropyridine receptor, one associated with activation of the channel and the other with inhibition (*see below*).

Other receptors are present on L-type Ca channels that bind phenylalkylamines, bepridil and benzothiazepines (Glossmann et al., 1983*b*; Murphy et al., 1983; Garcia et al., 1984; Ptasienski, McMahon & Hosey, 1985; Galizzi et al., 1986*a*; Reynolds et al., 1986*b*). The phenylalkylamines and bepridil appear to bind to a common receptor (Galizzi et al., 1986*a*), which is distinct from the DHP receptor. Benzothiazepines may bind to the phenylalkylamine/bepridil receptor (Murphy et al., 1983; Galizzi et al., 1986*a*) or to a separate receptor that is allosterically coupled to the phenylalkylamine receptor (Glossmann et al., 1983*b*, 1984; Goll, Ferry & Glossmann, 1984). Results from several laboratories have presented evidence that phenylalkylamines, bepridil and benzothiazepines competitively interact at L-type Ca channel receptors (Murphy et al., 1983; Galizzi et al., 1986*a*); however, the fact that the benzothiazepines stimulate DHP binding, while phenylalkylamines and bepridil usually inhibit DHP binding, might suggest the presence of one binding site for the benzothiazepines and another for phenylalkylamines and bepridil. In this regard, high and low affinity binding sites for phenylalkylamines have been demonstrated in membranes from cardiac, skeletal and smooth muscle and brain (Ptasienski et al., 1985; Reynolds et al., 1986*b*; Qar et al., 1987), and it has been suggested that these may be differentially associated with the benzothiazepine receptor (Reynolds, Snowman, & Snyder, 1986*a*).

Yet another receptor on L-channels has been identified which binds neuroleptics of the diphenylbutylpiperidine series such as fluspirilene and pimozide (Gould et al., 1983; Galizzi et al., 1986*b*). [These drugs differ from other drugs used for Ca channel studies in that, at relatively high doses, they act at D_2-dopamine receptors to exert neuroleptic effects (Leysen et al., 1977) and as anxiolytics when used at very low doses (Hassel, 1985)]. Recent studies have shown that the diphenylbutylpiperidine receptor on L channels is distinct from, but allosterically coupled to, the receptors for dihydropyridines and phenylalkylamines (Qar et al., 1987). In those instances where the number of binding sites have been measured, the DHP, phenylalkylamine, benzothiazepine and diphenylbutylpiperidine receptors appear to be present in a 1 : 1 : 1 : 1 stoichiometry (Barhanin et al., 1987; Galizzi et al., 1986*a*,*b*). Thus, there are at least three or four (or perhaps more) drug receptors on L-type Ca channels that are distinct but capable of allosterically interacting.

While L-type Ca channels in skeletal, smooth and cardiac muscle and many neurons appear to contain receptors which bind DHPs, phenylalkylamines, benzothiazepines, and perhaps the diphenylbutylpiperidines, an interesting recent development has been the demonstration in *Drosophila* brains of high affinity (0.2–0.4 nM) phenylalkylamine receptors that are associated with bepridil, diltiazem and diphenylbutylpiperidine receptors, but devoid of an associated high affinity DHP receptor (Pauron et al., 1987). Whether or not these receptors are associated with a functional L type or other Ca channel remains to be demonstrated. However, a recent study has shown that carrot cells contain a relatively high density of phenylalkylamine receptors that can mediate blockade of ^{45}Ca influx, and, like the situation in *Drosophila* brain, are associated with binding sites for diphenylbutylpiperidines, bepridil and diltiazem but not DHPs (Graziana et al., 1988). Yet other studies have shown phenylalkylamine-sensitive, but dihydropyridine- and benzothiazepine-insensitive, Ca channels in bone cells (Guggino et al., 1988; Wagner et al., 1988*b*). These latter Ca channels appear to play a role in bone resorption.

It is generally agreed that several factors can significantly influence the interaction of Ca channel effectors with their receptor sites. Allosteric interactions between drugs acting at the different receptor sites have been extensively described (*see* Glossmann & Ferry, 1985, for examples). The inter-

actions are complex and not fully understood. However, the interactions observed in biochemical experiments appear to have their counterparts under physiological conditions as well. An interesting example is the ability of D-*cis*-diltiazem, a benzothiazepine, to increase the binding of dihydropyridines to DHP receptors in isolated membranes (DePover et al., 1982; Bolger et al., 1983; Glossmann, Ferry, & Boschek, 1983*a*; Borsotto et al., 1984*a*; Kirley & Schwartz, 1984; Maan, Ptasienski, & Hosey, 1986) and to potentiate the action of certain dihydropyridines in physiological experiments (Dubé, Baik, & Schwartz, 1985). Bepridil, which binds to the same receptor as phenylalkylamines (Galizzi et al., 1986*a*), also has been reported to potentiate DHP binding and action (Balwierczak et al., 1986). A better understanding of these complex allosteric interactions will rely on a further elucidation of the properties of each of the receptor types present on L-type Ca channels.

Voltage Dependence of Dihydropyridine Action and Binding

After the early biochemical studies performed with radiolabeled DHPs demonstrated that these drugs bound to their receptor sites in isolated membranes with nM-pM affinities, it was thought that the affinities were too high compared to what was expected from physiological studies. This led to the concern that the biochemically identified DHP receptors might not correspond to functional Ca channels. This concern was largely eliminated by the demonstration that the actions and binding of dihydropyridines are strongly voltage dependent. Ca channel inhibitors of the DHP series act with significantly higher affinity on depolarized compared to polarized preparations (Bean, 1984; Sanguinetti & Kass, 1984; Cognard et al., 1986*b*; Sanguinetti, Krafte, & Kass, 1986; Hamilton et al., 1987; Kunze et al., 1987). Studies in cardiac muscle demonstrated that nitrendipine inhibited Ca channels in resting muscle with a $K_{0.5}$ of 700 nM, but with a $K_{0.5}$ of 0.36 nM in depolarized preparations (Bean, 1984). In skeletal muscle the $K_{0.5}$ for (+)PN 200-110 to inhibit Ca channels in depolarized cells was 0.15 nM, whereas the $K_{0.5}$ for (+)PN 200-110 to inhibit the same channels in polarized cells was significantly higher, 13 nM (Cognard et al., 1986*b*). These findings are generally interpreted to indicate that DHPs bind better to the inactivated form of the channel than to the resting form. (The "resting" form of the channel refers to a state in which the channel is closed but available to open, whereas the "inactivated" form refers to a state in which the channel is closed and cannot open. For a more detailed description of channel kinetics and mechanistic properties, *see* Hille, 1984.) In biochemical studies of DHP binding, high affinity binding has been usually observed because most studies have used isolated, depolarized membrane preparations. In the case of skeletal muscle, an excellent agreement was found between the K_d of 0.22 nM for (+)[^{3}H]PN 200-110 to bind to isolated (depolarized) skeletal muscle membranes and the $K_{0.5}$ (0.15 nM) for inhibition of channels in the depolarized cells (Cognard et al., 1986*b*). However, in another study, in adrenal medullary tumor cells, the IC_{50} values for DHPs to inhibit Ca channels in electrophysiological studies were significantly higher than the K_D values determined in binding studies, and the differences could not be eliminated by accounting for voltage-dependent effects (Kunze et al., 1987). Nevertheless, it is now generally agreed that there is a close relationship between high affinity DHP binding sites and functional Ca channels. Some data that further support this are that the conversion of high to low affinity DHP binding sites is response to differences in membrane potential has now been demonstrated in studies of DHP binding to intact cells and membrane vesicles (Green et al., 1985; Schilling & Drewe, 1986; Kamp & Miller, 1987). In addition, it has been shown that a good correlation exists between the number of high affinity DHP binding sites and the calculated number of functional Ca channels in clonal adrenal medullary cells (Kunze et al., 1987). Finally, it appears that DHP binding sites are only found in tissues known to contain L-type Ca channels and are absent in tissues devoid of these channels.

Ca Channel Receptors Are Also Modulated by Temperature and Divalent Cations

In addition to membrane potential, other factors also influence the interaction of drugs with receptors on Ca channels. The interactions of some Ca channel effectors with L channel receptors are significantly affected by temperature. Temperature-dependent effects for the interaction of D-*cis*-diltiazem with receptors in skeletal muscle membranes have been observed; the number of receptors that bind the ligand was fourfold higher at 2 than at 37°C (Glossmann et al., 1983*b*). Several Ca channel activators and inhibitors of the DHP series have been found to bind to cardiac membranes with 5-20 fold higher affinity at 4 than at 37°C (Maan & Hosey, 1987). Somewhat similar effects were observed with brain, smooth and skeletal muscle membranes (Rampe et al., 1987). These findings from biochemical studies agree well with certain ob-

servations from physiological studies; specifically, the reversal of Ca channel inhibition by DHP Ca channel inhibitors occurs more rapidly at 37°C than at lower temperatures (Rakowski, Olszewska & Paxson, 1987). Presumably the faster reversal occurs because of the decreased affinity and the more rapid dissociation which occurs at elevated temperature (Glossmann & Ferry, 1985; Maan & Hosey, 1987). The fact that DHPs dissociative *very* slowly from their receptors at 4°C had practical consequences in the purification of DHP receptors (discussed below). Temperature has also been shown to significantly influence the allosteric interactions between Ca channel effectors; for example, in skeletal muscle membranes, the phenylalkylamine (−)D888 stimulated the binding of the DHP (+)[^{3}H]PN 200-110 at 37°C, had no effect at 22°C, and inhibited binding at 0°C (Reynolds et al., 1986*a*).

Electrophysiological studies of the L-type Ca channel have provided evidence that the channel is a single-file pore that contains at least two high affinity (~1 μM) binding sites for Ca (Almers & McCleskey, 1984; Hess & Tsien, 1984; Hess, Lansman & Tsien, 1986; Lansman, Hess & Tsien, 1986). These high affinity Ca binding sites are believed to impart the selectivity of the Ca channel for Ca and to play an intimate role in the permeation of Ca through the channel. It is interesting then that the high affinity interactions of Ca channel effectors with their receptor sites on the L channel can be influenced in several ways by Ca and other divalent cations. The binding of DHPs to receptors in isolated membrane preparations is strictly dependent on the presence of a divalent cation (Glossmann et al., 1982; Gould, Murphy, & Snyder, 1982, 1984; Bolger et al., 1983; Luchowski et al., 1984; Ptasienski et al., 1985; Ruth et al., 1985; Maan et al., 1986; Maan & Hosey, 1987). Most results suggest that micromolar concentrations of divalent cations are required for DHP binding, while fairly high concentrations can be inhibitory. The requirement for divalent cations can be revealed through the use of the chelator EDTA to control the concentration of divalent cations present in membrane preparations or assay reagents. EDTA alone abolishes high affinity DHP binding, but binding can be fully restored by micromolar amounts of Mg^{2+}, Ca^{2+} or other divalent cations (Glossmann et al., 1982; Luchowski et al., 1984; Ptasienski et al., 1985). Divalent cations have also been found to affect phenylalkylamine binding to Ca channel receptors. Low concentrations of divalent cations influenced the relative proportions of high and low affinity phenylalkylamine receptors detected in chick cardiac membranes (Ptasienski et al., 1985). On the other hand, Ca inhibited phenylalkylamine, bepridil and benzothiazepine binding to skeletal muscle membranes with a K_d of 5 μM; other divalent cations also inhibited binding (Galizzi, Fosset, & Lazdunski, 1985) with the same rank potency order that they inhibit Ca flux through the Ca channel. These results could suggest that allosteric interactions may occur between the high affinity Ca binding sites in the channel protein and the receptors for Ca channel effectors.

Some Dihydropyridine Enantiomers Have Opposite Actions of Ca Channels

As previously mentioned, some DHP derivatives possess the ability to activate Ca channels. An interesting aspect of the pharmacology of certain DHPs concerns the opposing actions of several pairs of dihydropyridine stereoisomers: (−)Bay k 8644 and (+)202-791 are Ca channel activators, while (+)Bay k 8644 and (−)202-791 are Ca channel inhibitors (Frankowiak et al., 1985; Hof et al., 1985; Kongsamut et al., 1985; Williams et al., 1985; Uematsu et al., 1986; Kokubun et al., 1986; Wei et al., 1986; Hamilton et al., 1987). Not all DHP stereoisomer pairs exhibit these properties, as both enantiomers of PN 200-110 are channel inhibitors, although they differ dramatically in their affinities for the DHP receptor (Hof et al., 1984). The affinities with which the enantiomers of Bay k 8644 and 202-791 bind to their receptors and inhibit or activate the channel also are different; both (−)enantiomers are considerably more potent than their corresponding (+)enantiomers in physiological and biochemical tests (Franckowiak et al., 1985; Hof et al., 1985; Kongsamut et al., 1985; Williams et al., 1985; Uematsu et al., 1986; Hamilton et al., 1987; Maan & Hosey, 1987). It is unclear what key feature(s) determine whether a compound acts as an activator or inhibitor. No correlation exists between potency, or optical rotation, and whether or not a compound is an activator or inhibitor. A thermodynamic analysis performed in an attempt to determine if fundamental differences exist between the properties of activator and inhibitory ligands showed that the two "pure" activators tested, (−)Bay k 8644 and (+)202-791, differed from all other compounds analyzed in that the binding of the activators was driven entirely by enthalpy and was associated with unfavorable decreases in entropy (Maan & Hosey, 1987).

The finding that the stereoisomers of Bay k 8644 have opposite actions was originally proposed (Hof et al., 1985; Kongsamut et al., 1985) to explain the previously observed biphasic effects (stimulation at low concentrations and inhibition at high concentra-

tions) of racemic Bay k 8644 on the contraction of cardiac and smooth muscle preparations (Schramm et al., 1983). However, recent studies have found that, depending on the experimental conditions, the pure activators (−)Bay k 8644 and (+)202-791 can also produce stimulatory and inhibitory effects (Franckowiak et al., 1985; Williams et al., 1985; T.J. Kamp, M.C. Sanguinetti & R.J. Miller, *personal communication*). Other studies with the pure enantiomers have shown that the activator (+)202-791 allosterically modulated the effects of the inhibitor (−)202-791 and enhanced the binding of another DHP inhibitor, (+)PN 200-110, to intact cardiac cells (Kokubun et al., 1986). These results were explained by postulating that DHP receptors may contain two binding sites, one associated with activation and another with inhibition (Kokubun et al., 1986). This reasoning could explain the inhibitory and stimulatory effects of a pure isomer (Franckowiak et al., 1985; Williams et al., 1985), as well as the differences in the thermodynamic characteristics associated with the binding of activator and inhibitory ligands (Maan & Hosey, 1987). A somewhat similar reasoning was used to explain inhibitory and stimulatory effects of racemic nitrendipine and Bay k 8644 on Ca channels in guinea pig ventricle (Brown et al., 1986). However, the possibility that subtypes of DHP receptors exist which may mediate activation and inhibition of channel activity cannot be entirely ruled out.

New Ca Channel Effectors

It is likely that we will soon see a new series of chemically distinct molecules with actions towards Ca channels. One example is HOE 166 (Fig. 2). This benzolactam competitively and noncompetitively inhibits the binding of other Ca channel effectors to muscle membranes with high affinity (K_d = 0.25 nM) and blocks L-type Ca channels in both muscle and secretory cells (Qar et al., 1988). In addition to synthetic compounds active towards Ca channels, the demonstration that L type Ca channels contain numerous binding sites for chemically different Ca channel effectors suggest that endogenous molecules capable of affecting Ca channel activity may exist. Further suggestion that this might be so comes from studies on other types of channels which have shown that mammalian brain contains peptides which have functional (and in some cases structural) properties analogous to toxins that act on Ca-activated K channels (Fosset et al., 1984), voltage-dependent Na channels (Lombet et al., 1987) and voltage-dependent K channels (Cherubini et al., 1987).

Developmental Properties of L-Type Ca Channels

The ontogenic appearance of L-type Ca channels has been followed during the development of cardiac and skeletal muscle as well as the nervous system by quantitating the appearance of receptors for Ca channel effectors and DHP-sensitive ^{45}Ca efflux (Kazazoglou et al., 1983; Renaud et al., 1984). In chick skeletal muscle, for example, L-type Ca channels were not detected in myoblasts; they first appeared in myotubes and the number of DHP receptors increased further after innervation occurred (Schmid et al., 1984*b*). Denervation of muscle was found to produce a further increase in DHP receptors; this phenomenon was most likely due to the denervation-induced proliferation of the T-tubular membrane system (Schmid et al., 1984*a*). In the nervous system, autoradiographic techniques have shown that the ontogenetic appearance of Ca channels identified with the phenylalkylamine (−)D888 in mammalian brain is closely related to the development of dendritic arborization (Mourre, Cervera & Lazdunski, 1987).

Toxins and Natural Compounds as Pharmacological Probes of Ca Channels

Studies of other ion channels, particularly the nicotinic acetylcholine receptor-associated channel (Changeux et al., 1984) and voltage-dependent Na channels (Catterall, 1984), have been facilitated by the use of toxins which have specific and selective effects on channel function. The use of various neurotoxins provided numerous insights into detailed aspects of channel functions and also provided tools for the isolation, purification and eventual cloning of these ion channels. The success of these studies has prompted a search for toxins with specific and selective effects on Ca channels. The most interesting Ca channel toxin isolated to date is ω-conotoxin GVIA, a 27 amino acid peptide isolated from a mixture of neurotoxins present in the venom of the fish hunting marine snail *Conus geographus* (Olivera et al., 1984, 1985). This toxin, which was first shown to cause blockade of Ca entry into nerve terminals and irreversibly block the Ca component of the action potential in dorsal root ganglion neurons (Kerr & Yoshikami, 1984), has been demonstrated to cause persistant and direct inhibition of both N-type and L-type Ca channels in vertebrate neurons (Reynolds et al., 1986*b*; McCleskey et al., 1987) and an L-type Ca channel in clonal rat GH_3 pituitary cells (Suzuki and Yoshioka, 1987). However, ω-conotoxin does not affect all L-type channels, as it is without effect on L type-Ca channels in smooth,

cardiac and skeletal muscle (McCleskey et al., 1987). Nor does it affect all neuronal Ca channels, as T-type channels are insensitive to ω-conotoxin as are Ca channels in *Aplysia* bag-cell neurons (McCleskey et al., 1987). Different levels of inhibition by ω-conotoxin of Ca channels in synaptosomes derived from different species has been observed (Suszkiw, Murawsky & Fortner, 1987). Conceivably this could be due to different amounts of the susceptible channels.

The site of action of ω-conotoxin on neuronal L-channels is different from that of the 1,4-dihydropyridines, as there is no effect of dihydropyridines on the ability of ω-conotoxin to bind to its receptor sites or to cause Ca channel blockade (Cruz & Olivera, 1986; Abe et al., 1986; Rivier et al., 1987). ω-Conotoxin has been reported to inhibit Ca channels and bind to its receptor sites with pM-nM affinity (Reynolds et al., 1986*b*; Cruz & Olivera, 1986; Abe et al., 1987; Rivier et al., 1987). The dose-response relationships for channel inhibition appear complex, which conceivably reflects the interaction of the toxin with more than one channel type. However, a recent study using equilibrium binding and kinetic analysis determined that ω-conotoxin interacted with a single class of binding sites in chick and rat brain synaptosomes with a K_d of 0.8-2 pM (Barhanin, Schmid & Lazdunski, 1988).

The availability of ω-conotoxin in its iodinated form (Cruz & Olivera, 1986) has provided a potential tool for the isolation and biochemical characterization of N-channels. Since ω-conotoxin inhibits both N and L-type Ca channels, one would expect that the probe might identify more than one peptide component. However, recent studies have suggested that ω-conotoxin may preferentially identify N-channels, because the number of neuronal conotoxin binding sites was much higher than the number of DHP binding sites, and because the distribution of conotoxin binding sites throughout different brain areas did not parallel the distribution of DHP binding sites (Wagner et al., 1988*a*). Results of chemical cross-linking studies showed that the iodinated toxin specifically labeled components of 135,000-210,000 daltons in chick brain synaptosomes (Cruz, Johnson, & Olivera, 1987). Other studies have suggested that the target of 135 kDa was most likely derived from a peptide of 170 kDa which was cleaved to components of 140 and 30 kDa upon reduction of disulfide bonds (Barhanin, Schmid & Lazdunski, 1988). This peptide appears to be similar to a peptide that co-purifies with the muscle dihydropyridine receptor (*see below*). An azidonitrobenzoyl derivative of ω-contoxon, used as a photoaffinity probe of the conotoxin receptors in rat synaptic plasma membranes, labeled components of 310,000, 230,000 and 37,000 daltons (Abe & Saisu, 1987), and a peptide of 210-220 kDa in chick brain synaptosomes (Barhanin et al., 1988). Future studies should provide insight into the nature of the photolabeled components. An ω-conotoxin sensitive and dihydropyridine-insensitive Ca current has been expressed in *Xenopus* oocytes injected with mRNA from *Torpedo* electric lobe (Umbach & Gundersen, 1987). Whether or not the expressed channel is an N-type channel will require further clarification.

Other toxins have been characterized for their actions towards Ca channels. Maitotoxin, from the toxic dinoflagellate *Gambierdiscus toxicus*, was initially reported to be an activator of voltage-sensitive Ca channels because it was shown to: (i) produce a Ca-dependent contraction of smooth muscle; (ii) stimulate Ca-mediated release of hormones and neurotransmitters; and (iii) increase ^{45}Ca uptake into neuronal cells (Miyahara, Akau & Yasumoto, 1979; Takahashi, Ohizumi & Yasumoto, 1982; Ohizumi & Yasumoto, 1983; Freedman et al., 1984*b*; Kim, Login & Yasumoto, 1985). However, recent electrophysiological data indicate that the steady-state current induced by maitotoxin does not flow through voltage-dependent Ca channels (Yoshii et al., 1987). Beta-leptinotarsin-D and -H, polypeptide neurotoxins isolated from the hemolymph of Colorado potato beatles, also have been proposed to act at Ca channels because they stimulated Ca-dependent release of neurotransmitters from mammalian nerve terminals, pheochromocytoma cells and neuromuscular junctions (Yoshino, Baxter & McClure, 1980; Crosland, Hsiao & McClure, 1984; Madedder et al., 1985; McClure et al., 1985). A comparison of the characteristics of transmitter release evoked by depolarization or leptinotarsins led to the proposal that the toxins are direct activators of presynaptic voltage-sensitive Ca channels (McClure et al., 1985). Other toxins that are potential Ca channel activators include a polypeptide toxin that has recently been isolated from *Goniopora* coral (Qar et al., 1987), and atrotoxin, a polypeptide partially purified from snake venom (Hamilton et al., 1985). Another snake venom toxin, taicatoxin, has been reported to partially block L-type Ca channels in cardiac cells (Brown et al., 1987). In addition, tetrandine, a bis-benzoylisoquinoline alkaloid derived from a Chinese medicinal herb, has been found to block L-type Ca channels and appears to interact at the diltiazem binding site (King et al., 1988). It is likely that future studies will clarify whether these and other compounds do indeed act directly on Ca channels and will provide needed insight into their mechanisms of action.

Interestingly, two alkaloid toxins that are well

known activators of voltage-dependent Na channels, veratridine and batrachotoxin, were found to inhibit L-type Ca channels in neuroblastoma cells in the same range of concentrations as those required for their action on Na channels (Romey & Lazdunski, 1982). On the other hand, DHPs have been found to inhibit Na channels in a manner that is as specific and stereoselective as their effects on Ca channels, although the concentrations of DHPs required for Na channel inhibition are five- to 10-fold larger than for Ca channels (Yatani, Kunze & Brown, 1988). These actions of different agents on both Na and Ca channels may reflect homology between the voltage-dependent Na and Ca channels (*see below*).

At present, there is no specific pharmacology associated with voltage dependent T-type channels, although it seems likely that molecules with specific actions towards these channels will be eventually identified. Such molecules will be helpful in providing a further understanding of the role and properties of T-type Ca channels and to allow for a characterization of their molecular structure. It would not be surprising if the eventual isolation and cloning of the different types of voltage-dependent Ca channels reveals striking homologies in their structures.

Biochemical Characterization of L-Type Ca Channel

Only L-type, or DHP-sensitive Ca channels, have been well characterized biochemically. The success in characterizing L-type channels was largely due to the availability of high affinity, radiolabeled probes in the form of Ca channel effectors. In addition, much of the early success in characterizing and purifying this channel resulted from the use of skeletal muscle membranes as the sourse of the protein, as skeletal muscle transverse tubule membranes contain 50- to 100-fold more high affinity DHP receptors than any other source yet identified (Fosset et al., 1983; Glossmann et al., 1983*a*). The strategy utilized for the purification of L type channels was to purify the protein component(s) as high affinity DHP receptors, in hopes that the purified component(s) would constitute functional Ca channels. The probability for success with this approach was suggested by the previous successful purification of the nicotinic acetylcholine receptor associated ion channel and voltage-dependent Na channels as neurotoxin receptors (*see* Catterall, 1984; Changeux et al., 1984).

Studies using radiation inactivation and target size analysis gave initial estimates of molecular weights (M_r) of 185,000-278,000 for DHP receptors in brain, skeletal and smooth muscle (Ferry et al., 1983; Norman et al., 1983; Venter et al., 1983). A thorough hydrodynamic analysis of dihydropyridine receptors associated with cardiac Ca channels suggested a M_r of 370,000 after correction for bound detergent (Horne, Weiland, & Oswald, 1986*a*). The first report that a component of relatively high M_r might contain the DHP receptor came from studies performed with the DHP photoaffinity analog [^{3}H] azidopine, which labeled a 145-kDa component in guinea pig skeletal muscle membranes (Ferry et al., 1984). Shortly thereafter, convincing evidence that a large component contains the DHP receptor, as well as other receptors for Ca channel effectors, was provided by the results which demonstrated the specific photolabeling of a 170-kDa component of transverse tubule membranes prepared from skeletal muscle (Galizzi et al., 1986*a*). Three chemically different drugs, (+)[^{3}H]PN 200-110, [^{3}H]D-*cis*-diltiazem and (±)[^{3}H]bepridil, each labeled a seemingly similar component which migrated with an apparent M_r of 170,000 on SDS gels under reducing conditions (Galizzi et al., 1986*a*). Similar results have been obtained subsequently by a number of different laboratories using either the dihydropyridine [^{3}H]azidopine or the phenylalkylamine [^{3}H]azidopamil ([^{3}H]LU 49888) (Striessnig et al., 1986*b*, 1987; Hosey et al., 1987; Sieber et al., 1987; Sharp et al., 1987; Takahashi et al., 1987; Vaghy et al., 1987). The results suggest that a *single* peptide, which we will refer to as the 165-kDa peptide (but which migrates on SDS gels with an apparent M_r of 160-200,000 depending on the type of gel utilized; *see* Hosey et al., 1987), contains the high affinity receptors for at least four chemically distinct types of Ca channel inhibitors: the 1,4-dihydropyridines, the phenylalkylamines, the benzothiazepines and bepridil.

It should be noted that the 165-kDa peptide labeled by Ca channel effectors in skeletal muscle membranes is distinct from another protein of similar M_r (150,000-180,000), which has also been photolabeled by DHPs and is known as the multidrug-resistant protein. The phenomenon of multidrug resistance is a clinically important problem characterized by diminished sensitivity to multiple types of drugs. Multidrug-resistant cells possess a diminished capacity to accumulate therapeutic drugs compared to drug-sensitive cells. The multidrug-resistant protein (also known as P-glycoprotein) has been implicated as playing a key role in this phenomenon and is overexpressed in multidrug-resistant cells. A number of recent reports have described the ability of classical Ca channel inhibitors to reverse multidrug resistance (Tsuruo et al., 1981; Fojo et al., 1985; Beck et al., 1986; Cornwell et al.,

1986) and the multidrug-resistant protein has been shown to possess receptors for these drugs (Cornwell, Pastan, & Gottesman, 1987; Safa et al., 1987). The affinity of the multidrug-resistant protein for Ca channel effectors is considerably lower than receptors associated with L-type Ca channels (50 pM-1 nM for the Ca channel *vs.* > 100 nM for the multidrug-resistant protein). The DHP photoaffinity ligand azidopine has been shown to specifically label the multidrug-resistant protein in membranes of lung cells selected for resistance to vincristine or actinomycin D (Safa et al., 1987). However, the multidrug-resistant protein does not appear to be present in normal skeletal muscle membrane (Safa et al., 1987) and is clearly distinct from the 165-kDa putative Ca channel peptide in that cDNA cloning has shown that there is no homology between the two proteins (*compare* Tanabe et al., 1987 to Gros, Croop & Housman, 1986, and Chen et al., 1986).

Purification of the Dihydropyridine and Phenylalkylamine-Sensitive Ca Channel

Efforts to purify the DHP and phenylalkylamine-sensitive Ca channel were initiated using skeletal muscle transverse tubule membranes and either digitonin or CHAPS as the solubilizing agent (Curtis & Catterall, 1984; Borsotto et al., 1984*b*). The advantage of using CHAPS was that it was possible to perform direct binding studies with [^{3}H]PN 200-110 or [^{3}H]nitrendipine on the CHAPS solubilized material (Borsotto et al., 1984*b*). In contrast, when digitonin was used, it was usually necessary to prelabel the DHP receptors and to follow the fate of the label throughout the purification process (Curtis & Catterall, 1984). This was possible because of the very slow dissociation of the DHPs from their receptors at 4°C. Initial reports on the purification of the DHP receptor from skeletal muscle indicated that the purified fractions were enriched in a large polypeptide of 140–170 kDa and smaller polypeptides of 30–32 kDa (Curtis & Catterall, 1984; Borsotto et al., 1984*a*; 1985). The presence of a 55-kDa peptide was variably observed (Curtis & Catterall, 1984).

Recently it became evident that the purified preparations contained two dinstinct polypeptides of approximately 165-170 kDa. The heterogeneity of the ~170 kDa peptides, designated α_1 and α_2 by Striessnig et al. (1987), was demonstrated in studies using photoaffinity labeling, antibodies, and phosphorylation by cAMP-dependent protein kinase. One peptide of 165 kDa (α_1), but not the other, can be phosphorylated by cAMP-dependent protein kinase (Hosey et al., 1987; Imagawa, Leung & Campbell, 1987*a*; Takahashi et al., 1987). The M_r of this peptide varies slightly on different types of SDS gels (Hosey et al., 1987), and M_r values of 155,000–200,000 have been reported. Photoaffinity labeling studies (discussed above) showed that the phosphorylatable 165 kDa (α_1) peptide contains both the high affinity DHP and phenylalkylamine receptors (Sharp et al., 1987; Hosey et al., 1987; Sieber et al., 1987; Striessnig et al., 1987; Takahashi et al., 1987; Vaghy et al., 1987). Further evidence that the phosphorylatable 165 kDa (α_1) peptide is associated with the DHP receptor came from studies showing that two different monoclonal antibodies (Leung, Imagawa & Campbell, 1987; Morton & Froehner, 1987) and a polyclonal antibody (Takahashi & Catterall, 1987) were able to specifically immunoprecipitate the [^{3}H]PN200-100 labeled receptors from solubilized preparations and recognized the 165 kDa peptide in Western blots. A third monoclonal antibody, which activates DHP-sensitive Ca channels in skeletal muscle membranes, may also recognize the 165 kDa peptide (Malouf et al., 1987).

The second peptide of approximately 170 kDa present in purified preparation of DHP receptors and referred to as α_2 (Streissnig et al., 1987) can be differentiated from the 165 kDa (α_1) peptide by several criteria. The M_r of the 170 K (α_2) peptide decreases from 170,000 to 140,000 upon reduction with disulfide reducing agents (Curtis & Catterall, 1984; Hosey, Borsotto & Lazdunski, 1986; Schmid et al., 1986*a*), whereas the M_r of the 165 kDa (α_1) peptide is either unchanged or slightly increased under reducing conditions (*see* Streissnig et al., 1987; Vaghy et al., 1987; Hosey et al., 1987). The reduction-dependent decrease in the apparent M_r of 170 (α_2) is accompanied by the dissociation of the 170 kDa peptide into peptides of 140 and approximately 30 kDa (Schmid et al., 1986*a*,*b*; Barhanin et al., 1987). Other important distinctions between the 165 kDa (α_1) and the 170/140 kDa (α_2) peptides are that the 170/140 kDa peptide is not photolabeled by dihydropyridines or phenylalkylamines, is not recognized in Western blots by the monoclonal antibodies which immunoprecipitate DHP binding, and is not a substrate for cAMP-dependent protein kinase. The peptide maps of the 170 and 165 kDa peptides are different (Hosey et al., 1987; Sieber et al., 1987), and the 165 kDa peptide has been reported to be more hydrophobic than the 170 kDa peptide (Takahashi et al., 1987). It should also be noted that the 165 kDa peptide is much less stable than the 170 kDa peptide; this instability is the most likely reason that the 165 kDa peptide was not always observed in initial purification studies.

L-type Ca channels have also been purified from cardiac muscle. Since cardiac muscle contains 50- to 100-fold fewer DHP and phenylalkylamine

receptors, the purification of the L-type Ca channel from this tissue has been considerably more difficult. The first attempt to purify high affinity DHP receptors from chick cardiac muscle resulted in the purification of peptides of 60,000, 55,000 and 32,000 (Rengasamy, Ptasienski, & Hosey, 1985); however, proteolysis appears to have been a problem. A second study resulted in the purification of a larger peptide of 170 kDa from chick heart with characteristics similar to the α_2 purified from skeletal muscle (Cooper et al., 1987; Cooper, O'Callahan & Hosey, 1988). The 170 kDa α_2-like peptide purified from chick heart was recognized (Cooper et al., 1987) by polyclonal (Schmid et al., 1986*a*,*b*) and monoclonal (Vandaele et al., 1987) antibodies raised against skeletal muscle preparations. In terms of the peptide containing the dihydropyridine receptors, a 165 kDa peptide in guinea pig heart membranes has been photolabeled with [^{3}H]azidopine (Ferry, Goll & Glossmann, 1987). More recently, purification of the cardiac dihydropyridine/phenylalkylamine receptor has been achieved, and the results suggest that there are structural and immunological differences in the cardiac and skeletal muscle dihydropyridine/phenylalkylamine receptors (F.C. Chang & M.M. Hosey, *submitted for publication*). The cardiac DHP receptor co-purified with a 140 kDa peptide which appeared to be similar to the 170/140 kDa peptide (F.C. Change & M.M. Hosey, *submitted*). Future studies will aid in establishing the similarities and differences in the structures of L channel peptides purified from cardiac and skeletal muscle.

The purification of L-type channels from neuronal tissue has not yet been achieved. However, photoaffinity labeling studies have provided evidence that a high affinity phenylalkylamine receptor in *Drosophila* brains has a M_r of 135 kDa (Pauron et al., 1987). Furthermore, antibodies raised against the skeletal muscle 170/140 kDa peptide were used to demonstrate the presence and cytochemical localization of this peptide in rabbit brain (Schmid et al., 1986*b*). As this peptide has also been recently labeled with ω-conotoxin (Barhanin et al., 1988), future studies may help to discern whether it is a functional component of neuronal Ca channels.

The Dihydropyridine/Phenylalkylamine Receptor is Homologous with Voltage-Dependent Na Channels

Recently, the cDNA for the 165 kDa DHP receptor (α_1) from skeletal muscle has been cloned (Tanabe et al., 1987). The results predicted that the true M_r of the peptide is 210,00; it is not clear whether the 165 kDa peptide identified biochemically as the DHP receptor represents a proteolytically processed form of the DHP receptor, or whether its glycosylation and/or hydrophobicity contribute to aberrant electrophoretic behavior. Indeed, one group has reported the identification of a 210-220 kDa peptide, and this apparent high M_r may be due to the different gel system and M_r markers utilized (Morton & Froehner, 1987; Morton et al., 1988). An exciting finding from the cloning of the cDNA for the DHP receptor was that the predicted amino acid sequence of the peptide has striking homology to that of the voltage-dependent Na channels (Noda et al., 1984, 1986*b*). The overall homology of 29% identical residues is found throughout the protein and might explain why L-type Ca channels can behave as Na channels in the absence of Ca (Almers & McCleskey, 1984), why some neurotoxins that activate Na channels are also active toward L-type Ca channels (Romey & Lazdunski, 1982), and why DHPs can inhibit Na channels (Godfraind et al., 1986; Yatani et al., 1988). Previous studies established that voltage-dependent Na channels are comprised of a major polypeptide of 260 kDa (*see* Catterall, 1984) and that the mRNA derived from the cDNA for this peptide is sufficient to obtain expression of functional Na channels in oocytes (Goldin et al., 1986; Noda et al., 1986*a*; Stühmer et al., 1987). The predicted amino acid sequences of the skeletal muscle DHP receptor and the voltage-dependent Na channels possess four internal repeats, each of which is predicted to contain six membrane-spanning regions (Noda et al., 1984, 1986*a*; Tanabe et al., 1987). In each case, the fourth membrane-spanning segment in each internal repeat contains clusters of basic amino acids which have been suggested to comprise the voltage sensors (Tanabe et al., 1987; Noda et al., 1984, 1986*b*). The collected features of the 165 kDa peptide, particular the demonstrations that it contains many of the receptors for Ca channel active drugs *and* structurally resembles another protein known to constitute a functional ion channel, strongly suggest it comprises the major functional unit of L-type Ca channels. Functional studies with the expressed protein are needed to establish whether this is so.

Are There Subunits of L-Type Ca Channels?

The question as to whether or not there are subunits of the DHP/phenylalkylamine-sensitive Ca channel remains an issue. While it is clear that the 165 kDa (α_1) peptide contains the receptors for Ca channel effectors and may be the key functional component of L-type Ca channels, the role of the 170 (α_2) kDa

peptide is unknown. However, as noted above, the 170 kDa peptide copurifies with DHP receptors from rabbit and guinea pig skeletal muscle (Hosey et al., 1987; Leung et al., 1987; Sharp et al., 1987; Sieber et al., 1987; Streissnig et al., 1987; Takahashi et al., 1987; Vaghy et al., 1987) and from chick cardiac muscle (F.C. Chang & M.M. Hosey, *submitted*). This co-purification from different sources might suggest that the peptides are functionally related, but no unequivocal evidence for this has been obtained. Autoradiographic and immunofluorescent studies provided evidence that DHP receptors (presumably similar to the 165 kDa peptide) and a peptide reactive with antibodies raised against the skeletal muscle 170/140 kDa peptide are similarly distributed in different brain areas (Schmid et al., 1986*b*). Recent suggestions that the 170 and 165 kDa peptides were subunits of dihydropyridine-sensitive Ca channels were based on data concerning the glycosylation of the two peptides. It was suggested that the 170 kDa (α_2) peptide was a glycoprotein but the 165 kDa (α_1) peptide was not (Leung et al., 1987; Takahashi et al., 1987), and therefore it was proposed that the co-purification of the two peptides from a lectin affinity column resulted from their strong association. However, other data suggests that both the 170 and 165 kDa peptides are glycoproteins (Hosey et al., 1987) and several consensus sequences for glycosylation of the 165 kDa peptide are found in its N-terminal amino acid sequence (Tanabe et al., 1987).

In addition to the two large peptides, the presence of 55, 30–32, and 27–29 kDa peptides (designated as the β, γ, and δ subunits, respectively, Takahashi et al., 1987) have also been observed variably in some, but not all, purified preparations of DHP receptors. The nonglycosylated (Takahashi et al., 1987) 55-kDa (β) peptide has been identified as a substrate for cAMP-and Ca/calmodulin-dependent protein kinases and protein kinase C (Curtis & Catterall, 1985; Hosey et al., 1986; Nastainczyk et al., 1987). If this peptide is a true subunit of the channel, conceivably it could be involved in regulation of the channel by phosphorylation (*see below*). Several peptides of ~30 kDa have been observed in purified channel preparations. One 30–32 kDa (γ) peptide is not covalently associated with the 140-kDa peptide and has been shown to be heavily glycosylated (Takahashi et al., 1987). Peptide(s) of 25–29 kDa (δ), which are only observed in preparations electrophoresed under reducing conditions, normally covalently associate with the 140 kDa peptide to comprise the 170 kDa (α_2) peptide. These latter small peptides also have been shown to be glycosylated (Barhanin et al., 1987; Takahashi et al., 1987). More than one of these smaller peptides have been observed but appear to be derived from a common precursor as they give rise to similar peptide maps (Barhanin et al., 1987). The role of any of the ~30 kDa peptides is unknown. Whether they may be at all similar to the ~30 kDa peptides proposed to be associated with a neuronal Na channel (*see* Catterall, 1984) remains to be determined. In the latter case, it is clear that the small peptides are not necessary to obtain functional Na channel activity (Goldin et al., 1986; Noda et al., 1986*b*; Stühmer et al., 1987).

A problem in determining if any of these peptides are subunits is that the relative amounts of the different peptides in the purified preparations has been variable. This nonstoichiometric relationship could suggest that the peptides are not subunits, or it could be caused by a differential susceptibility of the peptides to proteolysis or to other factors which could cause subunit dissociation during purification. The strongest evidence suggesting that there are subunits of the L channel comes from immunoprecipitation studies with monoclonal antibodies. Several different groups of investigators have demonstrated co-immunoprecipitation of several peptides, including the 170/140, 165, 55 and 32 kDa peptides (Leung et al., 1987; Morton & Froehner, 1987; Leung et al., 1988) using anti-DHP receptor (165 kDa) antibodies. Furthermore, several of these peptides appeared to copurify during a purification step utilizing a monoclonal antibody immunoaffinity column (Malouf et al., 1987). In addition, monoclonal antibodies raised against the 55 (52) kDa peptide, which clearly contains no DHP binding sites, have been shown to immunoprecipitate DHP binding, and peptide mapping showed that the 55 kDa peptide was not proteolytically derived from the 165 kDa DHP receptor (Leung et al., 1988). Whether or not all of these potential "subunits" are necessary to constitute a functional Ca channel needs to be rigorously tested in either reconstitution or RNA expression studies. In this regard, DHP-sensitive Ca channels have been expressed in oocytes injected with crude mRNA from adult and neonatal rat hearts (Dascal et al., 1986; Moorman et al., 1987); however, successful expression of Ca channels with mRNA derived from the cDNA for the putative Ca channel components has not yet been reported.

Are Dihydropyridine/Phenylalkylamine Receptors in Skeletal Muscle and Neurons Functional Ca Channels?

It is well accepted that DHP-sensitive Ca channels in cardiac and smooth muscle are extremely impor-

tant in providing Ca to be used directly or indirectly for contraction. However, whether or not Ca channels and/or receptors for Ca channel effectors are involved in excitation-contraction coupling in skeletal muscle has been the subject of some debate, and several questions remain open to discussion. Skeletal muscle is not critically dependent on extracellular Ca as is cardiac or smooth muscle. Thus it has been somewhat of a paradox as to why skeletal muscle contains such a high number of high affinity receptors for Ca channel effectors (Fosset et al., 1983; Glossmann et al., 1983*a*) compared to other tissues in which Ca channels are clearly more important for function. Since the receptors for DHPs, phenylalkylamines, benzothiazepines and diphenylbutylpiperidines are all present in high density and in a 1 : 1 : 1 : 1 ratio in the transverse tubule membranes of skeletal muscle (Galizzi et al., 1987; Barhanin et al., 1987), it would seem logical that these receptors were associated with functional L-type Ca channels. However, it has been suggested that less than 5% of the high affinity DHP receptors correspond to functional voltage-dependent Ca channels in skeletal muscle (Schwartz et al., 1985). On the other hand, it has been suggested that DHP receptors may be involved in the process of excitation-contraction coupling in skeletal muscle in a capacity other than their potential involvement as components of voltage-dependent Ca channels (Rios & Brum, 1987). In this regard it has been proposed that the DHP receptor molecules may act as voltage sensors involved in the process of charge movement and eventual stimulation of Ca release from the sarcoplasmic reticulum (Rios & Brum, 1987). Despite these uncertainties, it is clear that DHP-sensitive Ca channels are present in skeletal muscle (Sanchez & Stefani, 1978; Nicola-Siri, Sanchez & Stefani, 1980; Potreau & Raymond, 1980; Almers & McCleskey, 1984; Scwartz et al., 1985; Cognard et al., 1986*a*,*b*; Rosenberg et al., 1986), and it is clear that preparations of purified DHP/phenylalkylamine receptors can constitute functional Ca channels (*see below*). Further studies are clearly warranted to improve our understanding of the physiological role of the Ca channel effector receptors and Ca channels in skeletal muscle. In addition, in view of the suggested possibility that DHP receptors in skeletal muscle may be *functionally* different from DHP receptors in heart and other tissues (Schwartz et al., 1985; Rios & Brum, 1987), it will be important to continue efforts to isolate putative L-type Ca channels from other tissues. A comparison of the properties of the isolated proteins may provide insight into their putative functional differences.

It should be underlined that the problem of the role of DHP and phenylalkylamine receptors and L-type Ca channels in the central nervous system is not much clearer. Varying concentrations of DHP and phenylalkylamine receptors have been identified in different regions throughout the brain (Cortes et al., 1984; Quirion, 1985; Mourre et al., 1987) and they would seem to correspond to functional channels (*see* Miller, 1987*a*). However, intracerebroventricular injections of DHPs or phenylalkylamines have been found to have little or no effect on electroencephalographic recordings (G. Gandolfo, J.N. Bidard, C. Gottesmann & M. Lazdunski, *unpublished results*). Moreover, while it is generally believed that Ca entry through voltage-dependent Ca channels appears to be an essential step in the coupling of excitation to neurosecretion at synapses, it is clear that L-type Ca channel inhibitors often have little or no effect on neurotransmitter release (*see* Godfraind et al., 1986; Miller, 1987*a*). Further evidence for this was provided by a recent study which demonstrated that N-type Ca channels, rather than L-type, play a dominant role in the evoked release of norepinephrine from sympathetic neurons (Hirning et al., 1988). However, it might be noted that even inhibition of N-type (and L-type) Ca channels with ω-conotoxin only partially inhibits neurotransmitter release (Reynolds, et al., 1986*b*; Hirning et al., 1988). The situation concerning the pharmacology of Ca channels that are involved in neurotransmitter release at synapses is in fact far from being clear. For example, although it is well-known that acetylcholine release at the neuromuscular junction is not sensitive to organic Ca channel blockers, a Ca current that can be induced after block of K channels is sensitive to verapamil and insensitive to ω-conotoxin (Anderson & Harvey, 1987). It is quite clear that much remains to be learned about the relationships between drug or toxin receptors believed to be associated with Ca channels and the roles of different Ca channels in excitation-contraction and -neurosecretion in muscle and nerve.

Reconstitution of Ca Channels

Several groups of investigators have reconstituted Ca channels from different sources. L-type (DHP-sensitive) Ca channels from skeletal and cardiac muscle have been have been incorporated into planar lipid membranes by fusion of membrane vesicles with the lipid bilayer (Affolter & Coronado, 1985, 1986; Ehrlich et al., 1986; Rosenberg et al., 1986). Comparative studies of the incorporated L-type channels from skeletal and cardiac membranes suggested that small but significant differences exist

in single channel conductance and gating in the channels from the two sources (Rosenberg et al., 1986). The properties of the channels incorporated into bilayers appeared to be similar to channels present in intact cells (Ehrlich et al., 1986; Rosenberg et al., 1986).

Since L-type Ca channels have been purified as high affinity DHP receptors, it was important to demonstrate that the purified preparations did indeed possess the ability to form functional DHP-sensitive Ca channels. Purified DHP receptor preparations from skeletal muscle have been reconstituted into planar lipid bilayers by several groups and shown to contain Ca channel activity (Flockerzi et al., 1986; Smith et al., 1987; Talvenheimo, Worley & Nelson, 1987). However, it is not yet clear which polypeptides are required to obtain Ca channel activity. The purified reconstituted channel possessed many characteristics in common with a DHP-sensitive Ca channel reconstituted directly (without purification) from skeletal muscle membranes and recorded under similar conditions (Smith et al., 1987). However, more than one type of conductance has been observed (Smith et al., 1987; Talvenheimo et al., 1987). Whether or not the different conductances represent different substates of a single Ca channel, or whether there is actually more than one Ca channel present in the purified preparations, has not yet been ascertained. The results obtained with purified reconstituted channels may very much depend on the experimental conditions, the method of reconstitution (Smith et al., 1987), the lipid composition of the bilayer (Coronado, 1987) and the peptide composition of the purified preparations. If there are indeed subunits of DHP-sensitive Ca channels, the relative amounts of the different peptides may effect the results obtained in reconstitution studies.

Dihydropyridine-sensitive Ca channels have also been incorporated into liposomes and the binding of Ca channel effectors (Horne et al., 1986*b*; Barhanin et al., 1987) and ^{45}Ca flux (Curtis & Catterall, 1986) measured. Studies with crude CHAPS extracts of skeletal muscle membranes showed that high affinity DHP receptors could be incorporated into liposomes and that the reconstitution stabilized the solubilized preparation (Horne et al., 1986*b*). The reconstituted DHP receptors were shown to be allosterically linked to phenylalkylamine/diltiazem receptors. Studies with purified preparations from skeletal muscle demonstrated reconstitution of receptors which bound dihydropyridines, phenylalkylamines and D-*cis*-diltiazem with high affinity and high specific activity (850–1260 pmol/mg protein) and in a 1:1:1 stoichiometry (Barhanin et al., 1987). While these studies suggested that the protein containing the receptors for the Ca channel active drugs could be successfully reconstituted to yield receptors with properties similar to those observed in native skeletal muscle membranes, the results of reconstitution studies in which ^{45}Ca flux through the channels was measured were somewhat paradoxical since less than 5% of the purified preparation appeared to be capable of mediating Ca flux (Curtis & Catterall, 1986). This finding could relate to the state of the preparation used (as discussed above), or to the possibility that not all the high affinity DHP receptors in skeletal muscle are components of functional Ca channels (Schwartz et al., 1985; Rios & Brum, 1987). Future studies with purified reconstituted preparations should prove helpful in further elucidating the properties of DHP-sensitive Ca channels obtained from different sources.

Regulation of Ca Channels by Neurotransmitter-Mediated Processes: Role of Cyclic AMP

Ca channels in many cells are subject to regulation by neurotransmitters by at least two different types of processes. The first to be recognized, and thus the more intensively studied, involves regulation of the channels by second messenger mediated phosphorylation/dephosphorylation dependent events. The second, more recently discovered, involves regulation of channel activity by GTP-binding proteins (G proteins).

The first Ca channel recognized to be regulated by neurotransmitters was the L-type Ca channel present in the heart. It has been appreciated for many years that norepinephrine, acting through beta-adrenergic receptors, increases the slow inward current (observed as the plateau phase of the cardiac action potential), while acetylcholine, via muscarinic cholinergic receptors, reverses the effect of norepinephrine and decreases slow inward current (for reviews, *see* Reuter, 1983; Sperelakis, 1984; Tsien et al., 1986; Reuter, et al., 1986). These effects of norepinephrine and acetylcholine are believed to involve cAMP-dependent regulation of the L-type Ca channels which are responsible for conducting the slow inward current. Any agent that elevates cyclic AMP in cardiac cells, including beta-adrenergic receptor agonists, modulates the activity of the cardiac L-type Ca channels by increasing their probability of opening (Reuter, 1983; Tsien et al., 1986). The effects of cAMP-elevating agents have been mimicked by microinjection of the purified catalytic subunit of cAMP-dependent protein kinase into isolated myocytes (Osterrieder et al., 1982; Brum et al., 1983) and inhibited by injection of

the specific cAMP-dependent protein kinase inhibitor (Bkaily & Sperelakis, 1984). Taken together, the results support the hypothesis that cardiac L channels are regulated by the cAMP-dependent phosphorylation of the channel itself or a regulatory protein. The inhibitory effects of activation of cardiac muscarinic receptors are most likely due to decreases in cAMP, decreased amounts of active cAMP-dependent protein kinase and net dephosphorylation of the regulated protein (Reuter, 1983). While good evidence exists to suggest that the muscarinic effects are due to decreased cAMP (Fischmeister & Hartzell, 1986; Hartzell & Fischmeister, 1986; Hescheler, Kameyama & Trautwein, 1986), other regulatory processes may be involved since acetylcholine is believed to regulate other second messenger systems in cardiac muscle (Brown & Brown, 1984).

L-type Ca channels in other cells also appear to be subject to regulation by cAMP-dependent protein kinase. L-type Ca channels in skeletal muscle (Schmid, Renaud & Lazdunski, 1985; Arreola et al., 1987), in newborn rat dorsal root ganglion neurons (Fedulova et al., 1985), in *Helix* neurons (Chad & Eckert, 1986), in N1E-115 neuroblastoma cells (Narahashi, Tsunoo & Yoshii, 1987) and in excitable cells derived from a rat pituitary tumor (GH_3 cells) (Armstrong & Eckert, 1987) are also positively modulated by processes that appear to involve cAMP-dependent phosphorylation. However, it is not clear whether all L-type Ca channels are regulated by cAMP; for example, L-type Ca channels in smooth muscle cells appeared to be insensitive to cAMP (Galizzi et al., 1987). While it is possible that the latter findings could reflect a true insensitivity of smooth muscle L-channels to cAMP, the possibility that the channels might have been fully activated by cAMP under the conditions studied cannot be entirely ruled out.

The evidence that cAMP-dependent phosphorylation of certain L channels is important in regulation of their activity comes largely from electrophysiological and ^{45}Ca flux studies (Reuter, 1983; Tsien et al., 1986). Data such as those cited above in reference to cardiac L channels, and those obtained from patch-clamp experiments in which the widely observed run-down of Ca channel activity over time could be restored by addition of cAMP-dependent protein kinase and ATP (Fedulova et al., 1985; Chad & Eckert, 1986; Armstrong & Eckert, 1987), all provide convincing evidence that the channels are regulated by a cAMP-dependent phosphorylation event. Nevertheless, a direct demonstration that peptides associated with L-type Ca channels are phosphorylated by cAMP-dependent protein kinase *in intact cells* has not yet been achieved. However, progress in elucidating the polypeptide composition of these channels has led to the demonstration that the 165 kDa peptide identified as the skeletal muscle DHP and phenylalkylamine receptor is a good substrate in vitro for cAMP-dependent protein kinase (Curtis & Catterall, 1985; Hosey et al., 1986, 1987; Imagawa et al., 1987*a*; Nastainczyk et al., 1987; O'Callahan & Hosey, 1988). In vitro phosphorylation studies have shown that the 165 kDa peptide can be multiply phosphorylated, primarily at serine residues, by cAMP-dependent protein kinase (O'Callahan & Hosey, 1988). The peptide can be phosphorylated in intact transverse tubule membranes (Hosey et al., 1986; O'Callahan & Hosey, 1988) or in solubilized, purified preparations (Curtis & Catterall, 1985; Hosey et al., 1987; Nastainczyk et al., 1987) with equal efficiency. In agreement with these findings, the predicted amino acid sequence derived from the cDNA for the 165 kDa peptide shows that the peptide contains six serines and one threonine in seven consensus sequences (Arg-Arg-X-Ser or Thr, *see* Krebs & Beavo, 1979) which could serve as substrates for cAMP-dependent protein kinase (Tanabe et al., 1987). All seven putative phosphorylation sites are predicted to be located cytoplasmically; six are contained in a hydrophilic C-terminal cytoplasmic tail, while the seventh is located on a short cytoplasmic loop connecting the second and third internal repeats (Tanabe et al., 1987). Although it is not certain that a 55 kDa peptide is a true subunit of DHP-sensitive Ca channels, phosphorylation of the 55 kDa peptide that copurifies with the 165 kDa peptide has been observed in in vitro studies with cAMP-dependent protein kinase (Curtis & Catterall, 1985; Hosey et al., 1986; Imagawa et al., 1987*b*; Nastainczyk et al., 1987; Takahashi et al., 1987). Whether or not phosphorylation of this peptide has functional effects on Ca channel function is not known. Future studies should elucidate which of the putative phosphorylation sites in the 165 kDa, and perhaps the 55 kDa, peptides are indeed phosphorylated in the intact cell and contribute to activation of the channels. In addition, as progress is made in purifying L-type channels from other sources, it should be interesting to determine if the phosphorylation sites are similar or different, and why some L channels are regulated by cAMP while others are not.

In addition to the short-term effects of cAMP on L-type Ca channels, cAMP also controls the synthesis of receptors for Ca channel effectors and functional L-type channels. Long-term treatment (48 hr) of chick skeletal myotubes with the β-adrenergic agonist isoproterenol, phosphodiesterase inhibitors, or cAMP analogs led to a large increase in

the level of DHP receptors (Schmid et al., 1985). Similar results were obtained with neuronal clonal cell lines (Freedman et al., 1984*a*; Nirenberg et al., 1984). In these latter cells, little or no L-type Ca channels were detected when the cells were grown under standard tissue culture conditions. However, when the intracellular content of cAMP was increased, the cells acquired functional voltage-dependent Ca channels that were sensitive to DHPs. This effect of cAMP on the expression of voltage-dependent Ca channels in neurons has been associated with a possible role of cAMP in the regulation of synaptogenesis (Nirenberg et al., 1984).

Although cAMP effects have been most thoroughly studied on L-type Ca channels, other types of Ca channels may also be positively or negatively regulated by cAMP-dependent processes. N and T-type Ca channels in neurons appear to be insensitive to cAMP (Fedulova et al., 1985; Narahashi et al., 1987; Wanake et al., 1987); however, cAMP has been reported to have stimulatory effects on fast (T-type) Ca channels in frog skeletal muscle (Arreola et al., 1987) and guinea pig cardiac myocytes (Mitra & Morad, 1986). Certain Ca channels in *Helix* neurons (Brezina, Eckert & Erxleben, 1987) have been shown to be insensitive to cAMP, while two apparently different receptor-operated Ca channels in a clonal osteosarcoma cell line appear to possess differential sensitivity to cAMP in that one is activated while the other is inhibited by cAMP (Yamaguchi et al., 1987*a*).

Protein Kinase C Inhibits Some Ca Channels and Activates Others

Some Ca channels are regulated by protein kinase C (Kaczmarek, 1986). Activators of protein kinase C, such as tumor promoting phorbol esters and synthetic diacylglycerols, have been shown to *inhibit* voltage-dependent Ca channels in embryonic chick dorsal root ganglion neurons (Rane & Dunlap, 1986; Marchetti & Brown, 1988), PC12 pheochromocytoma cells (DiVirgilio et al., 1986; Harris, Kongsamut & Miller, 1986), insulin secreting RINm5F cells (DiVirgilio et al., 1986), certain *Helix aspera* neurons (Hammond et al., 1987), GH_3 anterior pituitary cells (Marchetti & Brown, 1988) and A7r5 aortic smooth muscle cells (Galizzi et al., 1987) (however, stimulatory effects of phorbol esters on Ca channels in A7r5 cells have also been reported; *see* Sperti & Colucci, 1987). While some of these effects of protein kinase C activation appear to be due to effects on L-type channels, the effects of protein kinase C may involve regulation of other channels. For example, in chick dorsal root ganglion neurons and the GH_3 cells, protein kinase C activators inhibited both L and T currents (Marchetti & Brown, 1988). That phorbol esters primarily cause inhibition of N type currents in mouse dorsal root ganglion neurons has been suggested (Gross & Macdonald, 1987).

Yet other types of Ca channels are *activated* by a protein kinase C mediated event. In *Aplysia* bag cell neurons, protein kinase C activation leads to the appearance of Ca channels not observed in unstimulated cells (De Riemer et al., 1985; Strong et al., 1987; Kaczmarek, 1986). These effects were unusual in that other modulations of Ca channels usually result from an activation or inhibition of a current observed under control conditions. One type of Ca channel in an osteosarcoma cell line also has been suggested to be activated by protein kinase C; this Ca channel appeared to be similar to a parathyroid hormone activated Ca channel in these cells (Yamaguchi, Kleeman & Muallem, 1987*b*).

As is the case with cAMP-dependent regulation of Ca channels, most evidence for regulation of Ca channels by protein kinase C comes from electrophysiological studies. However, with respect to the regulation of L-type Ca channels by protein kinase C, it has recently been demonstrated that the 165 kDa DHP/phenylalkylamine receptor in skeletal muscle can be phosphorylated in vitro by purified protein kinase C. Although one study suggested that the purified 165 kDa peptide was not a good substrate for protein kinase C (Nastainczyk et al., 1987), another has shown that the membrane-bound form of the 165 kDa peptide is efficiently phosphorylated by protein kinase (C.M. O'Callahan & M.M. Hosey, *submitted*). Whether or not phosphorylation of this peptide by protein kinase C is related to the functional effects caused by activators of protein kinase C needs to be determined. Little is known concerning effects of protein kinase C on L-type Ca channels in skeletal muscle, although one report has suggested that protein kinase C, like cAMP (Schmid et al., 1985), may regulate the appearance of high affinity DHP receptors in these cells (Navaro, 1987).

Other Protein Kinases May Also Regulate Ca Channels

The skeletal muscle DHP receptor can be multiply phosphorylated in transverse tubule membranes by a multifunctional Ca/calmodulin-dependent protein kinase (Hosey et al., 1986; Cooper et al., 1988). Phosphorylation occurs to an equal degree on threonine and serine residues and is additive with phosphorylation catalyzed by cAMP-dependent protein kinase (O'Callahan & Hosey, 1988).

Whether or not phosphorylation by Ca/calmodulin-dependent protein kinase has functional consequences has not yet been tested. Cyclic GMP-dependent protein kinase has been reported to activate a Ca current in *Helix aspera* neurons (Paupardin-Tritsch et al., 1986). This effect of cGMP-dependent protein kinase was mimicked by serotonin treatment or by inhibitors of cGMP-dependent phosphodiesterase (Paupardin-Tritsch et al., 1986). Thus, modulation of different Ca channels by at least four types of protein kinases has been observed in various preparations. Further studies are required, particularly at the biochemical and molecular level, to understand precisely how phosphorylation of either the channels themselves or associated regulatory proteins results in modification of channel behavior.

Dephosphorylation of Ca Channels Reverses Effects of Phosphorylation

As phosphorylation of certain Ca channels by protein kinases has been shown to modulate channel activity, dephosphorylation by phosphoprotein phosphatases would be expected to reverse the effects. It is not certain if complete dephosphorylation of those Ca channels regulated by phosphorylation leads to their complete inactivation or not. While it has been proposed that L-type Ca channels need to be phosphorylated to be active (Armstrong & Eckert, 1987), L-type channels that have been reconstituted from membranes or purified preparations have been found to be active in the absence of phosphorylating conditions (*see* references in section on reconstitution).

The effects of several different types of phosphoprotein phosphatases on Ca currents in cardiac cells and *Helix* neurons have been investigated. Perfusion of guinea pig ventricular myocytes with the catalytic subunits of either phosphoprotein phosphatase 1 or 2A reversed the ability of the β-adrenergic agonist isoproterenol to increase Ca current through L-type Ca channels (Kameyama et al., 1986; Heschler et al., 1987*a*). However, potato acid phosphatase and calf intestinal alkaline phosphatase were without effect (Heschler et al., 1987*a*). Perfusion of *Helix* neurons with the Ca/calmodulin-dependent phosphatase calcineurin resulted in an increased rate of inactivation of Ca channels previously exposed to phosphorylating conditions with cAMP-dependent protein kinase (Chad & Eckert, 1986). In biochemical studies, calcineurin was shown to effectively dephosphorylate the skeletal muscle 165-kDa DHP receptor which had been previously phosphorylated by either the cAMP-dependent or the multifunctional Ca/calmodulin-dependent protein kinase (Hosey et al., 1986). Taken together, these results suggest that calcineurin and phosphatases 1 and 2A may effectively reverse the effects of cAMP- and/or Ca-dependent phosphorylation of L-type Ca channels in intact cells. Studies concerning the effects of phosphoprotein phosphatases on other Ca channels have not been reported, but undoubtedly will be forthcoming and should further our understanding of the mechanisms by which phosphorylation and dephosphorylation modulate Ca channel activity.

Regulation of Ca Channels by G-Proteins

A relatively new development in the field of ion channels has been the realization that certain ion channels, including Ca channels, can be regulated by various G proteins (reviewed by Dunlap et al., 1987). The G proteins involved in the regulation of Ca channels appear to be similar to the G proteins that regulate the adenylate cyclase system (Gilman, 1987) and voltage-dependent K channels (Codina et al., 1987; Logothetis et al., 1987; Yatani et al., 1987*b*). Inhibition of voltage-dependent Ca channels by receptors coupled to G-proteins has been demonstrated with norepinephrine and $GABA_b$ receptors in chick (Holz, Rane & Dunlap, 1986) and rat (Scott & Dolphin, 1986) dorsal root ganglion neurons, with somatostatin receptors in the AtT-20/D16-6 pituitary cell line (Lewis, Weight & Luini, 1986), with opiate receptors in NG108-15 neuroblastoma X glioma hybrid cells (Hescheler et al., 1987*b*), with receptors for an endogenous neuropeptide in neurons of *Aplysia californica* (Brezina et al., 1987) and with muscarinic receptors in sympathetic neurons dissociated from neonatal rat superior cervical ganglion (Wanke et al., 1987). In the chick dorsal root ganglion neurons (Holz, Rane & Dunlap, 1986), the pituitary cells (Lewis, Weight & Luini, 1986), the neuroblastoma cells (Heschler et al., 1987*b*), and the rat sympathetic neurons (Wanke et al., 1987), the G protein involved was pertussis toxin sensitive, suggesting it was similar or identical to G_i or G_o (Sternweis & Robishaw, 1984; Gilman, 1987). Evidence for the participation of the alpha subunit of G_o in the opiate receptor regulated Ca channels in the neuroblastoma cells was obtained by infusing purified G_o or its alpha subunit into cells in which the endogenous G_o and G_i were inactivated by pertussis toxin treatment (Heschler et al., 1987*b*). In these experiments G_o was 10 times more effective than G_i in restoring opiate receptor-mediated inhibition of Ca channels. Whether or not G_o, which is highly abundant in neu-

ronal tissue (Sternweis & Robishaw, 1984), mediates the receptor/G-protein mediated inhibition of Ca channels in other cells has not yet been demonstrated.

A question that arises in these experiments is whether the inhibition caused by activation of G proteins occurs as a result of a direct interaction of the G protein with the Ca channel, or is indirect and involves the participation of a second messenger. Evidence favoring the former possibility has been presented. For example, in many cases, the involvement of cAMP has been eliminated (Forscher & Oxford, 1985; Oxford & Schulz, 1986; Holz et al., 1986; Lewis et al., 1986; Brezina et al., 1987; Wanke et al., 1987), and in others, roles for cGMP, Ca, inositol trisphosphate and/or protein kinase C (Forscher & Oxford, 1985; Brezina, et al., 1987; Wanke et al., 1987) have also been eliminated. One other result that argues for the lack of involvement of second messengers is that in chick dorsal root ganglion neurons norepinephrine applied outside the patch pipette (and theoretically able to modulate second messenger production) was largely ineffective in inhibiting Ca channels (Forscher et al., 1986). Further studies will be necessary to establish the mechanism(s) involved in the G-protein dependent inhibition of the above mentioned Ca channels. In addition, the identification of the type of Ca channel subject to inhibition by pertussis toxin sensitive G proteins also needs to be more firmly established. In sympathetic neurons derived from neonatal rat superior cervical ganglion the muscarinic receptor/G-protein mediated inhibition of Ca current was shown to be due to a selective inhibition of N-type Ca channels (Wanke et al., 1987). Although it appears that an L-type channel is inhibited by neurotransmitter-activated G protein(s) in chick dorsal root ganglion neurons (Holz et al., 1986; Marchetti, Carbone & Lux, 1986), it is possible that both L and N channels contributed to the currents recorded. In mouse dorsal root ganglion neurons, opiates acting on a κ-opioid receptor were shown to selectively inhibit N channels whereas L and T channels were largely unaffected (Gross & Macdonald, 1987). Whether this inhibition of N currents involved a G protein and/or was similar to the inhibition of Ca channels in chick dorsal root ganglion by norepinephrine (Forscher & Oxford, 1985; Forscher et al., 1986; Holz et al., 1986; Marchetti et al., 1986), $GABA_b$ (Deisz & Lux, 1985; Holz et al., 1986; Scott & Dolphin, 1986), dopamine (Marchetti et al., 1986) and adenosine (Dolphin, Forda & Scott, 1986) receptors remains to be established.

More recently *stimulation* of Ca channels by G proteins has been observed. Quite unexpectedly, the cardiac L channel has been shown to be directly activated by G_s (the G protein that stimulates adenylate cyclase) or its alpha subunit (Yatani et al., 1987*c*). In this case, convincing evidence was obtained to show that the effect of the G protein appeared to result from a direct activation of the L channels and was independent of second messenger production or activation of protein kinases. However, as the activation of the cardiac L channel by G_s occurred to a significantly greater extent with channels preactivated by the β-receptor agonist isoproterenol or the Ca channel activator Bay k 8644, it may be that physiologically, G_s serves to activate those Ca channels already activated by a phosphorylation-dependent event (Yatani et al., 1987*c*). Whether or not G proteins are involved in the neurotransmitter-mediated activation of other Ca channels, such as the acetylcholine-mediated activation of Ca channels in smooth muscle cells (Clapp et al., 1987), has yet to be demonstrated.

Obviously, the number of different Ca channels regulated by various mechanisms involving many different types of neurotranmitter and hormone receptors, second messengers, protein kinases and G proteins is far more complicated than was reflected from the original studies of regulation of cardiac Ca channels by norepinephrine and acetylcholine. With the rapid progress being made in molecular biological approaches to understanding ion channel function, we can look forward to new and exciting developments in elucidation of the complex mechanisms underlying the regulation of the various types of Ca channels in different cell types.

Defects in Ca Channels Are Related to Several Pathological States

As we have seen, modulation of Ca channels can occur at many levels. Disruption at any of these could have serious consequences, particularly in those situations in which Ca channels are of crucial importance in supplying Ca for normal cell function. Pathological states could likely be produced by: (i) any alteration of Ca channel structure, and hence of the biophysical properties, of Ca channels, (ii) any alteration of the numerous modes of regulation of Ca channels (through protein kinases, phosphatases or G proteins), and/or (iii) any alteration of the relationship of Ca channel proteins with the membrane environment. Ca channels and cardiovascular pathologies have been closely associated for a long time. In fact, drugs used in cardiovascular diseases such as hypertension, angina pectoris and arrhythmias have been essential in establishing our present knowledge of the molecular properties of L-type Ca channels. On the other hand, the results of

some molecular investigations of the mechanisms of action of DHPs have therapeutic implications. In particular, the observation that the DHPs have better affinity for more depolarized cells can be considered clinically relevant when one considers that diseased cells will, in general, be more depolarized than healthy ones and will therefore be more selectively affected by DHPs. In addition to their link to cardiovascular problems, a potential linking of Ca channels to pathologies of the nervous system has been recently realized in that neuroleptic drugs such as fluspirilene and pimozide are now recognized to also be potent inhibitors of L-type Ca channels (Galizzi et al., 1987).

Several types of diseases have been shown to be associated with abnormal Ca channel function. One is a hereditary cardiomyopathy of the hamster in which a progressive necrosis mimics human forms of cardiac hypertrophy. Lesions have been found to be associated with Ca overload and can be prevented by the phenylalkylamine verapamil. The number of binding sites for Ca channel inhibitors, as well as the activity of related voltage-dependent Ca channels, was found to be markedly increased in cardiomyopathic hamsters (Wagner et al., 1986). These results make it tempting to speculate that these increases may be involved in the pathogenesis of this cardiomyopathy. A second case of Ca channel associated pathology corresponds to a hereditary skeletal muscle disease. In the genetically dysgenic mouse, *mdg/mdg*, skeletal muscles do not contract. Therefore, birth cannot occur and the disease must be studied at the fetal stage. Skeletal muscles from fetal *mdg/mdg* mice have been shown to lack receptors for Ca channel effectors (Pincon-Raymond et al., 1985), as well as functional L-type Ca channels (Beam, Knudson & Powell, 1986; Romey et al., 1986), whereas the number of DHP receptors, as well as Ca channel function, appeared to be normal in cardiac muscle and the central nervous system (Beam et al., 1986; Romey et al., 1986). The disappearance of skeletal muscle Ca channels in the mutant was associated with the absence of the normal triadic structure which appears to be essential for normal channel expression in skeletal muscle (Pincon-Raymond et al., 1985). Normal triads were replaced by pseudotriads. Innervation of *mdg/mdg* skeletal myotubes in vitro with spinal cord neurons from normal mice was found to restore Ca channel activity, muscle contraction, and the appearance of normal triadic structures (Rieger et al., 1987). These later results clearly show that the defect in Ca channels in this disease is not due to a mutation of the Ca channel gene.

A third type of disorder associated with Ca channels is the Lambert-Eaton myasthenic syndrome (Fukunoya et al., 1983; Kim & Neher, 1988). This is an acquired neuromuscular disorder in which there is insufficient release of acetylcholine at the neuromuscular junction in response to a depolarization (Lambert & Elmqvist, 1971). It has now been proposed that this autoimmune disease appears to be due to circulating antibodies against Ca channels (Fukunoya et al., 1983; Kim & Neher, 1988). Autoantibodies from patients with this disease were tested for their effects on Ca channels in cultured bovine adrenal chromaffin cells. The IgG from six affected patients caused a reduction in the number of functional Ca channels without altering the properties of the unaffected Ca channels (Kim & Neher, 1988). Serum from control patients had no effects. As the antibodies tested were active toward L-type Ca channels, these results may suggest a role for L-type Ca channels in neurotransmitter release.

Other instances of abnormal Ca channel activity have been observed. Since abnormally high levels of cholesterol are well known to represent a risk factor in cardiovascular diseases, it is interesting that a dramatic decrease in the cholesterol content of cardiac cells was found to be associated with a drastic diminution of L-type Ca channel activity (Renaud et al., 1986). This altered channel activity was associated with a major decrease, and eventual total supression, of contractile activity. Finally, there are some indications that alterations in Ca channel activity may be related to alterations in cardiac function that occur during senescence. In the aging rat heart, a decreased responsiveness to catecholamines has been observed which may be related to some yet undefined alteration in the regulation of L-type Ca channels (Lakatta, 1985). Further investigations into this and other problems should provide new insights into the roles of Ca channels in disease and aging. With the rapid progress being made towards furthering our understanding of the molecular pharmacology, structure and regulation of Ca channels, it is anticipated that the results of future studies will lead to significant improvements in the treatment and prevention of disorders related to Ca channel function.

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