*REVIEW*

# Molecular Pharmacology of Voltage-Dependent Calcium Channels

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ABSTRACT—Voltage-dependent $Ca^{2+}$ channels serve as the only link to transduce membrane depolarization into cellular $Ca^{2+}$-dependent reactions. A wide variety of chemical substances that have the ability to modulate $Ca^{2+}$ channels have been demonstrated both for their clinic utility and for importance in elucidating the molecular basis of various biological responses. Recently, introduction of molecular biology to pharmacology has brought a great deal of information about the molecular basis of drug action in $Ca^{2+}$ channels. In this review, we attempt to overview recent progress in understanding the interactions between $Ca^{2+}$ channels and their blockers, namely $Ca^{2+}$ antagonists, from a molecular and structural point of view.

*Keywords*: $Ca^{2+}$ channel, $Ca^{2+}$ antagonist, Peptide toxin, Inorganic blocker

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## I. Introduction

Voltage-dependent $Ca^{2+}$ channels serve as the only link to transduce depolarization into activities controlled by excitation in diverse cellular processes including muscle contraction, neurosecretion and gating of other channels. The basis of this pivotal role of $Ca^{2+}$ channels is the transient increase of intracellular $Ca^{2+}$ concentration evoked by the gating of the channels. This allows exit from Hodgkin's classical cycle of electrical excitation where electricity gates channels, and channels generate electricity (1). $Ca^{2+}$ channels, together with $Na^+$ and $K^+$ channels, also have an electrogenic role, constituting the electrical cycle. Because of the physiological importance of $Ca^{2+}$ channels, essentially, all pharmacological agents that modulate $Ca^{2+}$ channel activity cause certain types of biological responses. A wide variety of chemical substances that have $Ca^{2+}$ channel-modulating ability have been demonstrated both for their clinic utility, and for importance in elucidating the molecular basis of various biological responses. $Ca^{2+}$ channel blockers have made the greatest contribution to purification and biochemical identification of $Ca^{2+}$ channel complexes from excitable tissues such as the skeletal muscle and brain. Moreover, in general, their pharmacological sensitivity has been proven to be a distinguishing criterion, by far more reliable than biophysical parameters, for classifying multiple types of $Ca^{2+}$ channels that control specific biological functions. This is due to the intrinsic nature of these drugs in precisely probing specific three-dimensional structures formed on the protein targets at the recognition (or binding) site. It may, therefore, not be surprising that the difference in the pharmacological specificity of $Ca^{2+}$ channel types correspond to their difference in molecular structure.

Recently, much information has been accumulating with regards to the molecular basis of drug action. In particular, the introduction of molecular and structural biology to pharmacology, has dramatically enhanced the understanding of structure-function relationships of drug receptor proteins and peptidergic drugs. This trend has not been exceptional for voltage-dependent $Ca^{2+}$ channels and their blockers, i.e., $Ca^{2+}$ antagonists. In this review, we will attempt to review recent progress in understanding interactions between $Ca^{2+}$ channels and their blockers from a molecular and structural point of view. We have been of necessity selective in literature citation and apologize for any omissions.

## II. Distinguishing criteria for functional classification of $Ca^{2+}$ channels

Establishing criteria for distinguishing among the multiple types of $Ca^{2+}$ channels is a prerequisite for elucidation of molecular mechanisms that underlie diverse cellular processes. Both biophysical and pharmacological criteria are usually employed in identifying the type of channel involved in a particular physiological process (2, 3). It has been recognized that there exist at least four types of $Ca^{2+}$ channels (L-, N-, P- and T-type) that can be distinguished by their functional properties such as time- and voltage-dependent kinetics, single-channel conductance and sensitivity to different pharmacological agents (Table 1) (3–7). The L-type $Ca^{2+}$ channels are high voltage-activated, sensitive to dihydropyridines (DHPs) and show a single-channel conductance of 22–27 pS. They are found in virtually all excitable tissues and in many non-excitable cells. They trigger excitation-contraction (E-C) coupling in skeletal muscle, heart and smooth muscle and control hormone or transmitter release from endocrine cells and some neurons. The N-type channel is largely restricted to neurons and is irreversibly blocked by ω-conotoxin (ω-CgTx)-GVIA (5, 7). In addition, ω-CgTx-GVIA inhibits transmitter release in a variety of mammalian neuronal preparations (8–11), thus supporting the hypothesis (12) that influx of $Ca^{2+}$ through N-type

Abbreviations used are (in alphabetical order): ω-Aga, ω-agatoxin; BHK, baby hamster kidney: BZT, benzothiazepine; ω-CgTx, ω-conotoxin; CHO, Chinese hamster ovary; Cys, cysteine; DHP, dihydropyridine; DPBP, diphenylbutylpiperidine; E-C coupling, excitation-contraction coupling; FTX, funnel-web spider toxin; ω-GsTx, ω-grammotoxin; HEK, human embryonic kidney; HIT, hamster insulin-secreting tumor; HoTx, *Hololena* toxin; HVA, high voltage-activated; LVA, low voltage-activated; *mdg*, muscular dysgenesis; PAA, phenylalkylamine; PLTX, *Plectreurys* toxin; Po, opening probability; Ser, serine.

Abbreviations for drugs and other chemicals (in alphabetical order): Bay K 8644, methyl 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylate; CGP 28392, ethyl-4-[2-(difluoromethoxy)phenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxofuro[3,4-*b*]pyridine-3-carboxylate; D888, 2,7-dimethyl-3-(3,4-dimethoxyphenyl)-3-cyan-7-aza-9-(3-methoxyphenyl)-nonane hydrochloride; D890, 5-(3,4-methoxyphenethyldimethylamino)-2-(3,4,5-trimethoxyphenyl)-2-isopropyl-valeronitrile; FPL 64176, methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1*H*-pyrrole-3-carboxylate; HOE 166, *R*-(+)-3,4-dihydro-2-isopropyl-4-methyl-2-[2-[4-[4-[2-(3,4,5-trimethoxyphenyl)ethyl]piperazinyl]butoxy]-phenyl]-2*H*-1,4-benzothiazine-3-on-dihydrochloride; LU49888, (−)-5-(3-azidophenethylmethylamino)-2-(3,4,5-trimethoxyphenyl)-2-isopropyl-valeronitrile; RS 30026, methyl 2-methyl-4-(2-benzyloxyphenyl)-5-oxo-1,4,5,7-tetrahydrothieno[3,4-*b*]pyridine-3-carboxylate; SDZ 207-180, 10-[[4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-[(1-methylethoxy)carbonyl]-3-pyridinyl]carbonyloxy]-*N,N,N*-trimethyldecanaminium chloride; SQ 32.428, (*cis*)-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-3-methyl-6-(trifluoromethyl)-1-[2-(dimethylamino)ethyl]-2*H*-1-benzazepine-2-one; 202-791, isopropyl 4-(2,1,3-benzoxadiazo-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridine-carboxylate.

**Table 1.** $Ca^{2+}$ channel toxins

| Toxin | | Source | Structure | Blocking selectivity |
|---|---|---|---|---|
| ω-Conotoxin-(CgTx) | GVIA | *Conus geographus* | CKSOGSSCSOTSYNCCRSCNOYTKRCY | N-type |
| | GVIIA | | CKSOGTOCSRGMRDCCTSCLLYSNKCRRY | |
| | MVIIA | *Conus magus* | CKGKGAKCSRLMYDCCTGSCRSGKC | N-type |
| | MVIIB | | CKGKGASCHRTSYDCCTGSCNRGKC | |
| | MVIIC | | CKGKGAPCRKTMYDCCSGSCGRRGKC | N, P, Q-type |
| | MVIID | | CQGRGASCRKTMYNCCSGSCNRGRC | |
| | SVIA | *Conus striatus* | CRSSGSOCGVTSICCGRCYRGKCT | |
| | SVIB | | CKLKGQSCRKTSYDCCSGSCGRSGKC | |
| ω-Agatoxin-(Aga) | IA | *Agelenopsis aperta* | AKALPPGSVCDGNESDCKCYGKWHKCRCPWKW--HFTGEGPCTCEKGMKHTCITKLHCPNKAEWGLDW | |
| | G | | EKGLPEGAECDGNESDCKCAGQWIKCRCPWKWH--ITGEGPCTCERGLKKTCISKLSCPNRNGW | |
| | IIA | | GCIEIGGDCDGYQEKSYCQCCRNNGFCS.... | |
| | IIIA | | SCIDIGGDCDGEKDDCQCCRRNGYCSCYSLFGYLKSG--CKCVVGTSAEFQGICRRKARQCYNSDPDKCESHINKPKRR | L, N-type |
| | IVA | | KKKCIAKDYGRCKWGGTPCCRGRGCICSIMGTNCE--CKPRLIMEGLGLA | P, Q-type |
| | IVB | | EDNCIAEDYGKCTWGGTKCCRGRPCRCSMIGTNCEC--TPRLIMEGLSFA | P, Q-type |
| ω-Grammotoxin-(GsTx) | SIA | *Grammostola spatulata* | DCVRFWGKCSQTSDCCPHLACKSKWPRNICVWDGSV | non-selective |
| PLTX-II | | *Plectreurys tristes* | ADCSATGDTCDHTKKCCDDCYTCRCGTPWGANCRC--DYYKARCDT | *Drosophila* $Ca^{2+}$ channels |
| Calciseptine | | *Dendroaspis polylepsis polylepsis* | RICYIHKASLPRATKTCVENTCYKMFIRTQREYISERG--CGCPTAMWPYQTECCKGDRCNK | L-type |
| Calcicludine | | *Dendroaspis angusticeps* | WQPPWYCKEPVRIGSCKKQFSSFYFKWTAKKCLPF--LFSGCGGNANRFQTIGECRKKCLGK | L, N, P-type |

$Ca^{2+}$ channels controls neurotransmitter release. Additionally, N-type $Ca^{2+}$ channels also appear to play a role in directed migration of immature neurons (13). The P-type $Ca^{2+}$ channels, first identified in Purkinje cells (6, 14), are found in a variety of neurons (15). They are high voltage-activated (HVA) $Ca^{2+}$ channels selectively blocked by ω-agatoxin-IVA (ω-Aga-IVA) (15) or funnel-web spider toxin (FTX) (14), but are insensitive to both DHPs and ω-CgTx-GVIA. These channels probably play an essential role in inducing long term depression in cerebellar Purkinje neurons. The T-type $Ca^{2+}$ channels are known as low voltage-activated (LVA) $Ca^{2+}$ channels that inactivate rapidly (4, 5, 16). These channels are relatively sensitive to $Ni^{2+}$, amiloride and octanol, but they are resistant to DHP and ω-CgTx-GVIA. They have a single channel conductance of ~8 pS. T-type channels have been implicated in repetitive firing and pacemaker activity in heart and neurons.

Despite the wide recognition of the validity of the T, N, L or P categories, numerous studies have demonstrated that $Ca^{2+}$ currents of a particular pharmacological specificity vary considerably in their biophysical properties, and it may not be possible to assign unequivocally a measured $Ca^{2+}$ current to particular types based solely on biophysical parameters (17–23). In initial descriptions, the inactivating and non-inactivating components of HVA $Ca^{2+}$ current were assigned to N-type and DHP-sensitive L-type channels, respectively (4, 5). However, more recent work has demonstrated that in many cells, ω-CgTx-GVIA blocks both inactivating HVA $Ca^{2+}$ current and a part of the non-inactivating HVA $Ca^{2+}$ current that is insensitive to DHP (17, 18, 20, 23, 24) and that the extent and rate of inactivation varies considerably among ω-CgTx-GVIA-sensitive channels in various neuronal cells (3). Moreover, the N- and L-type channels are hardly distinguishable on the basis of channel mean open time, in the absence of DHP agonists, voltage-dependence of inactivation and unitary conductance (20 and 25 pS, respectively) (24), although a requirement of deeper hyperpolarization to deactivate and smaller single-channel conductance compared to L-type channels was originally important hallmarks of N-type channels (17–19). Thus, selective responses to certain pharmacological agents are now recognized as the major critera to distinguish T, N, L and P categories of $Ca^{2+}$ channels.

## III. $Ca^{2+}$ channel blockers and their interaction with $Ca^{2+}$ channels

### A. Organic $Ca^{2+}$ channel blockers

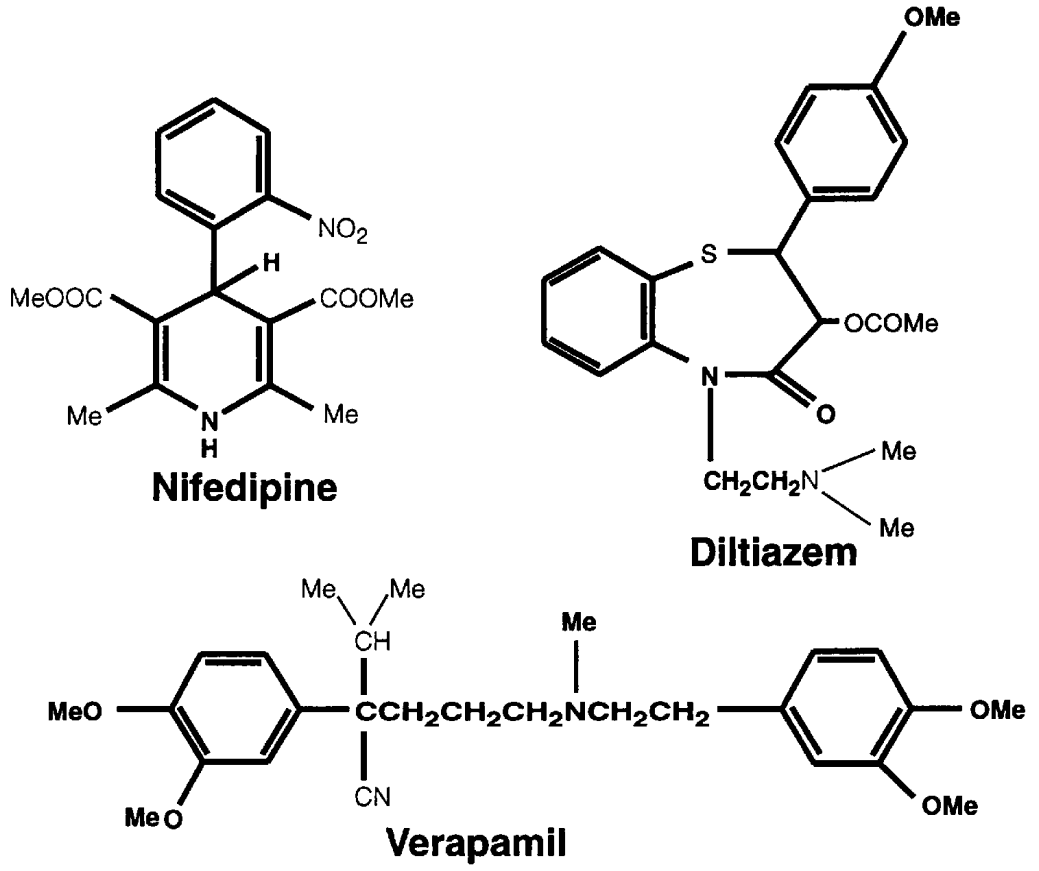
The drugs that modulate $Ca^{2+}$ channel activity, $Ca^{2+}$ channel blockers, were originally developed as coronary vasolidators. The later recognition of their negative ionotropic and dromotropic effects in the myocardium (25) led to an expansion in therapeutic applications. A variety of diseases treated by these drugs include hypertension, angina, certain forms of cardiac arrhythmias, congestive heart failure, hypertrophic cardiomyopathy, atherosclerosis, as well as cereberal and peripheral vascular disorders. Fleckenstein (26) was the first to report that the actions of verapamil and other phenylalkylamines involves inhibition of transmembrane $Ca^{2+}$ movements. They used the term "$Ca^{2+}$ antagonist" to classify these organic compounds as a new group of drugs, because the inhibitory actions of the drugs were antagonized by the concentration of extracellular $Ca^{2+}$. However, together with the gradual clarification of their direct inhibitory action on $Ca^{2+}$ channel currents, it became more common to call the drugs "$Ca^{2+}$ channel blockers". In addition to the major contributions $Ca^{2-}$ channel blockers have made to the management of clinical disorders, these drugs have been equally important towards the basic understanding of the functional behavior and biochemical properties of voltage-dependent $Ca^{2+}$ channels.

The three major classes (27) of organic blockers are: DHP (e.g., nifedipine), benzothiazepines (BZTs; e.g., diltiazem) (28) and phenylalkylamines (PAAs; e.g., verapamil) are quite dissimilar in molecular structure (Fig. 1). Binding experiments using radiolabeled ligands revealed that these drugs have separate but allosterically-linked binding sites on the L-type channels in muscle cells (29). $Ca^{2+}$ occupies a central role in the interaction of the three drugs, because $Ca^{2+}$ binds to the $Ca^{2+}$ channel and modulates, positively or negatively, the binding of the drugs. Thus, the term "$Ca^{2+}$ antagonist" is suitably used to describe the characteristics of these compounds. This pharmacological relationship of the three classes of drugs is described in detail in the following section. Although the three major $Ca^{2+}$ antagonists are the most important of the organic blockers, we will not go into further detail to precisely describe their pharmacological action, because numerous extensive reviews have already been written on their subject (29, 30).

In addition to the three major classes of $Ca^{2-}$ antagonists, there exist a vast number of organic molecules that show DHP-sensitive L-type $Ca^{2-}$ channel-modulating activity. A six-membered benzolactam, HOE 166, and its analogs are also high-affinity $Ca^{2+}$ antagonists (31). Decrease in apparent affinity by the bezolactams, without change in maximum binding capacity ($B_{max}$) and dissociation constant ($K_d$) of the DHP antagonist PN200-110 binding to skeletal muscle microsomes, indicates competitive interaction of the compounds at the DHP binding site in L-type $Ca^{2-}$ channels. In contrast, a decrease in $B_{max}$ by HOE 166 was observed for the PAA and BZT binding. A small but significant difference between benzolactams and DHPs in their effects was that HOE 166 became even more potent than PN200-110 in blocking $Ca^{2+}$ currents of smooth musle cell lines at hyperpolarized potentials. The results, togther with the existence of both agonist and antagonist in the DHP series, may imply that the DHP receptor $Ca^{2+}$ channel has separate recognition sites (or conformations) for the DHP ring structure and the chemical residues responsible for the agonistic and antagonistic action of the DHP drugs.

Diphenylbutylpiperidines (DPBPs), a group of neuroleptic drugs including fluspirilene, penfluridol and pimozide, are among the most potent inhibitors of the skeletal muscle $Ca^{2+}$ channels (32). Independent of the membrane potential, fluspirilene inhibited whole cell L-currents in skeletal muscle cells with half-maximal inhibition at concentrations of 0.1–0.2 nM. Binding of radiolabeled fluspirilene to T-tubule membranes was inhibited by the other DPBPs and non-DPBP neuroleptics including chloropromazine and haloperidol. Fluspridine noncompetitively inhibited the binding of the PAA derivative (−)D888, suggesting a DPBP site separate from that of PAA in the L-type $Ca^{2+}$ channel. Inhibition of $Ca^{2+}$ channel currents by chloropromazine has been indeed reported, although this drug also blocked $Na^{+}$ channels with similar affinity (33).

DHP agonists such as (−)-(*S*)-Bay K 8644 (34), 202-791 (35), CGP 28392 (36), RS 30026 (37) and other compounds have been studied for their enhancing effect on $Ca^{2+}$ channel activity. The benzoyl pyrrole derivative FPL 64176 (38) has an agonistic effect on L-type $Ca^{2+}$ channels, dramatically increasing the current amplitude (39), slowing current activation and inactivation, and prolonging tail current duration of the whole-cell patch clamp $Ca^{2+}$ current in smooth musle cells in a reversible manner (40). These kinetic effects of FPL 64176 were canceled by further addition of the DHP agonist (−)-Bay K 8644, but FPL 64176 failed to exert an effect on (−)-Bay K 8644 action. The contractile responses of smooth musle cells induced by the benzoyl pyrrole agonist were noncompetitively antagonized by the $Ca^{2+}$ antagonists nifedipine (DHP), verapamil (PAA) and dilitiazem (BZT). In fact, FPL 64176 is a weak inhibitor of the radiolabeled DHP, PAA and BZT binding (41). Dissociation kinetics of the radiolabeled derivatives of the three $Ca^{2+}$ antagonists were accelerated, and, consequently,



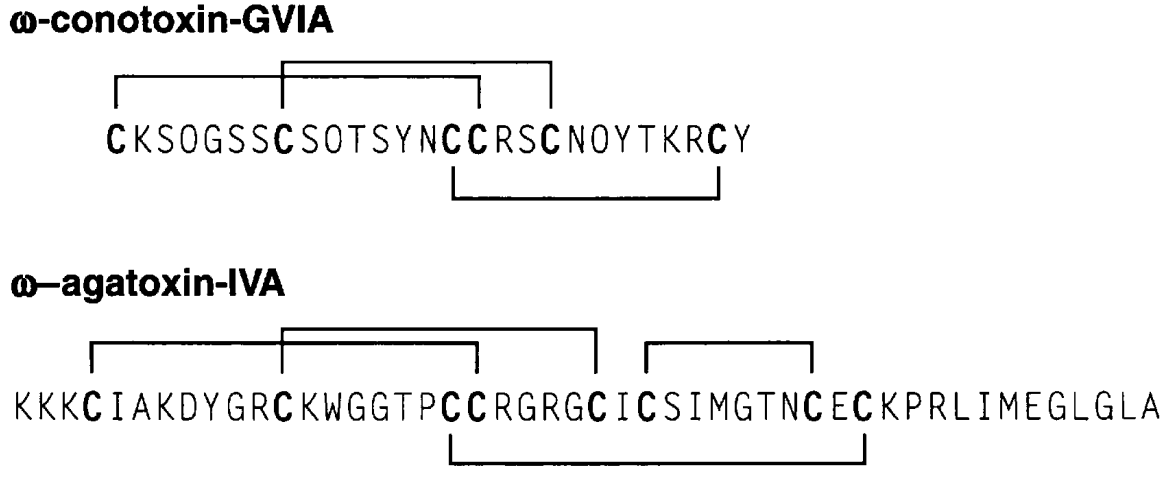




**Fig. 1.** Molecular structures of the major $Ca^{2+}$ antagonists and peptide toxins.

the binding affinities were significantly reduced with small and nonsignificant changes in $B_{max}$. Taken together, these reports suggest the negative allosteric interactions among the three major $Ca^{2+}$ antagonists and FPL 64176 at distinct sites or conformations in the $Ca^{2+}$ channel (probably the $\alpha_1$-subunit).

Another class of organic blocker is the polyamines and their derivatives. FTX, isolated as a fraction with a molecular mass in the 200- to 400-Da range from *Agelenopsis aperta* venom, was originally reported as a

specific blocker of P-type channels in Purkinji cells (14) and the ω-CgTx-GVIA- and DHP-resistant $Ca^{2-}$ channel currents expressed from rat brain mRNA in *Xenopus* oocytes (42). An affinity gel conjugated with the FTX fraction or synthetic FTX was used to biochemically purify the P-type channel. The nonreduced protein showed a molecular weight of ~120 kDa on size-exclusion high-performance liquid chromatography and 90–100 kDa on sodium dodecyl sulfate-polyacrylamide gel elctrophoresis, whereas upon reduction, at least two subunits were evident (70 and 20–30 kDa) (43). These molecular weights are by far lower than those deduced from the cDNA sequences of the neuronal $\alpha_1$-subunit isoforms that ranged from approximately 240 to 270 kDa (44). The isolated protein reconstituted in lipid-bilayers exhibited HVA channel openings that showed little or no inactivation and single channel conductances of 10–12 pS and 18–20 pS using 80 mM $Ba^{2+}$ as a charge carrier (45). A variety of chemical analyses performed on FTX suggests that it may be a nonaromatic polyamine (43, 45). A series of compounds they were synthesized based on spermidine, and were tested for their $Ca^{2+}$ channel blocking ability. Coupling of arginine to spermidine or substitution with triamines for spermidines resulted in a substantial enhancement of blocking activity. Another class of polyamines, putrescine, has been demonstrated to stimulate the L-type $Ca^{2+}$ channel without any effect on the T-type channel in neuroblastoma cells (46). This is in consistent with the correlation of elevated putresine levels and the progressive acccumulation of $Ca^{2+}$ with the density of injured neurons due to ischemia. The action of putrescine could be indirect through a second messenger system mediated via protein kinase C. The higher polyamines, spermidine and spermine, were without effect on the current of the L-type channel in neuroblastoma cells.

The dinoflagellate *Gambierdiscus toxicus* produces a highly toxic polyether, maitotoxin. This molecule is a unique toxin because it does not inhibit but activate L-type $Ca^{2+}$ channels by increasing their opening probability (Po) and increases the intracellular $Ca^{2+}$ concentration (47). The action site of this toxin is directly on the $Ca^{2+}$ channel as evidenced by its allosteric modulation of DHP binding (48).

Different classes of organic compounds such as tetramethrin (49), amiloride (50), diphenylhydantoin (51) and octanol (52) have been suggested to block T-type $Ca^{2+}$ channels. However, a lack of selectivity in their blockade of T-type channels has discouraged biochemical analyses of this type in efforts to reveal the molecular entity of T-type channels. Moreover, no laboratory has succeeded in isolating and recombinantly expresssing T-type cDNA, despite a great deal of effort.

## B. Peptide toxins

### 1. ω-Conotoxins and ω-agatoxins

Two classes of polypeptide toxins, referred to as ω-toxins (Fig. 1), have attracted considerable attention, because of their potency for inhibiting different biological activities through blockade of voltage-dependent $Ca^{2+}$ channels. ω-CgTx is one ω-toxin that has been isolated from different species of *Conus* snails, and ω-Aga, another toxin has been isolated from the funnel-web spider *Agelenopsis aperta*. Both toxins, potent $Ca^{2+}$ channel blockers, are produced in venom glands to paralyze and capture their more agile prey. These toxins, with their high affinity and selectivity to specific $Ca^{2+}$ channel types (Table 1), have proven to be powerful pharmacological probes for exploring the molecular bases of the diversity of $Ca^{2+}$ channels.

Marine snails of the genus *Conus* (piscivorous and molluscivorous snails) produce a set of small, disulfide-rich, $Ca^{2+}$ channel-blocking peptides, the ω-conotoxins (53, 54). The toxins were originally identified through biological activity assays using mainly fish and mice. All the ω-conopeptides cause paralysis and death in fish and molluscs, natural preys of different Cone snails. The effects on mice are usually more complex and are different among ω-conotoxins with different $Ca^{2+}$ channel subtype specificities (21, 54, 55). One of the typical symptoms is the "shaker" syndrome, which is induced upon intracerebral injections of toxins including ω-CgTx-GVIA and ω-CgTx-MVIIA. As the dose was increased, the "shaking" generally became more intense, earlier in onset, and longer in duration, but death never occurred. The in vivo biological effects of ω-CgTx-MVIIC were distinct from the other two ω-conotoxins (21). Intracerebroventricular injection of ω-CgTx-MVIIC was lethal and caused the gasping/arched back syndrome at low doses, such as 0.1–0.4 μg, less than 1/100 the ω-CgTx-MVIIA dose causing the shaker syndrome but not lethality in mice.

Amino acid sequencing of purified peptides, together with molecular cloning, has revealed a number of structural features shared by all ω-conotoxins (Table 1). A prepropeptide precursor of ω-CgTx, approximately 70 amino acids in length, is posttranslationally processed to the mature form of 24–29 amino acids by cleavage of the conserved N-terminal regions (56). In all natural mature ω-conotoxins so far, the C-terminus is amidated. The characteristic arrangement of cysteine (Cys) residues common among the ω-conotoxins is the so-called "four-loop Cys scaffold" (Fig. 1), which is not exclusively specific to $Ca^{2+}$ channel blocking peptides but also found in non-$Ca^{2+}$ channel blocking peptides such as "King-Kong peptides" (57). The native configuration of disulfide

bonds is apparently crucial for the ω-toxins to elicit the blocking effect on $Ca^{2+}$ channels. On the other hand, however, the loops between Cys residues are hypervariable, the only conserved non-Cys residue among ω-conotoxins being Gly 5. Interestingly, considerable divergence in amino acid sequences in the loop regions of the toxin, which are primarily responsible for the binding to $Ca^{2-}$ channels, is permissible for proper targeting without altering the $Ca^{2+}$ channel target specificity.

The American funnel-web spider *Agelenopsis aperta* is the only spider species whose venom have been biochemically analyzed in comprehensive detail (54, 58). Similar to the *Conus* venom in containing additional classes of paralytic peptides such as the α-conotoxins and μ-conotoxin that inhibit nicotinic acetylcholine receptors and voltage-dependent $Na^{+}$ channels, respectively, the spider venom is the source of blocker toxins that target sites other than $Ca^{2-}$ channels, including the acylpolyamine inhibitors of glutamate receptors, α-agatoxins, and the peptide activators of neuronal $Na^{+}$ channels, μ-agatoxins. The agatoxins are a heterogeneous group of polypeptides with a molecular mass of 5–10 kDa screened through three different bioassays, block of neuromuscular transmission in housefly body muscle (ω-Aga-I and ω-Aga-II) (54), inhibition of ω-CgTx-binding to chick synaptosomal membrane (ω-Aga-III) (59), and inhibition of $^{45}Ca^{2+}$ entry into both chick and rat synaptosomes (ω-Aga-IV) (60). cDNA cloning studies and amino acid sequencing of the purified preparations of the toxins have provided structural information about the ω-agatoxins (Table 1). ω-Agatoxins are more diverse than the ω-conotoxins in their primary structures, although they are similar in being abundant in Cys residues (8–12 residues) (Fig. 1). The precursor ω-Aga-IA, which contains a signal sequence and two cleaved glutamate-rich regions, is processed to the mature form with two chains connected by disulfide bonds (54). ω-Aga-IVA and ω-Aga-IVB are the best characterized $Ca^{2+}$ channel-blocking peptides in the spider venom. These 48-amino acid peptide toxins are homologous in structure (71% amino acid identity) (61, 62), having the same disulfide connectivity. Two forms of ω-Aga-IVB which differ in absolute configuration at serine (Ser) 46 (the corresponding residue is glycine in ω-Aga-IVA) have been found in the venom (61, 62). The D-Ser-containing isoform, which has been posttranslationally converted from the L-Ser isoform by the isomerase, has 80- to 90-fold more potency in the blockade of P-type and resistance to the major protease in the venom. Another petide toxin, Aga-GI, has been isolated from *A. aperta* venom. Aga-GI blocked the non-activating plateau phase of transient intracellular $Ca^{2+}$ increase in cerebellar granule cells, which is resistant to blockade by ω-CgTx-GVIA and ω-Aga-IVA (63), consequently inhibiting glutamate release from the granule cells. Biochemical properties of the toxin have not been clarified yet.

The two-dimensional $^{1}H$-NMR technique has been used to elucidate the three-dimensional structure for ω-CgTx-GVIA, as well as that for another group of peptide toxins, ω-Aga-IVA and ω-Aga-IVB (64–67). Irrespective of differences in the number of disulfide bonds and relatively low homology in primary structure, the toxins share structural characteristics, being composed of a short triple-stranded β-sheet and several reverse turns. In both groups of toxins, patches of positively charged residues or, in the case of ω-CgTx-GVIA, tyrosine residues with hydroxy residues are distributed on the molecular surface. These residues may contribute to the receptor-binding surface of the toxin molecules.

### 2. Other toxins

Isolation of toxins that antagonize $Ca^{2+}$ channel activity from venoms of other spider species have been reported (Table 1). In the search for potent toxins that inhibit neuronal $Ca^{2+}$ channels in *Dorosophila* with much less sensitivity to major toxins such as ω-CgTx-GVIA than those of vertebrates (68), presynaptic inhibitory activity was purified from the venom of the hunting spider *Hololena curta* (68) and *Plectreurys tristes* (69, 70), using *Drosophila* neuromuscular junction as an assay. Both *Hololena* toxin (HoTx) (~50 nM) and *Plectreurys* toxin (PLTX) (~10 nM) rapidly blocked $Ca^{2+}$ currents measured in cultured neurons from dissociated embryos, although the two toxins differed in the extent of the block, the former eliminating only the non-inactivating component of the $Ca^{2+}$ current and the latter inhibiting both the inactivating and non-inactivating component of the current (69, 71). The purified HoTX is composed of two different subunits of Mr 7,000 and 8,000 (68). HPLC purification and structural determination of one member of the PLTX family, PLTX-II, has revealed that PLTX-II is a 44-amino acid peptide with an *O*-palmitoyl threonine amide residue at its carboxy terminus (70). It is suggested that fatty acylation is required for the biological activity of the toxins, including PLTX-II because cleavage of the *O*-palmitoyl ester from the native toxin by base treatment results in a loss of biological activity.

The venom from the tarantula *Grammostola spatulata* is the source of a 36-amino acid toxin, ω-grammotoxin (ω-GsTx) SIA, which contains multiple disulfide bridges (72). The toxin completely inhibited rat and chick synaptosomal $^{45}Ca^{2+}$ influx, which is blocked mainly by ω-Aga-IVA (60) and completely by ω-CgTx-GVIA, respectively, suggesting a broad range of $Ca^{2+}$ channel blocking ability of ω-GsTx-SIA. Interestingly, ω-CgTx-GVIA-, PN 200-110- or desmethoxyverapamil-binding to neuronal

membrane fragments was not inhibited by the toxin (73).

Calciseptine is the first peptide that has been reported for L-type-selective blockade (74). This 60-amino acid peptide toxin, isolated from the venom of black mamba *Dendroaspis polylepis polylepis*, abolished both cardiac cell contraction and smooth muscle contraction, similar to the DHP antagonists. Similar activity was reported for the major 60-amino acid component of the same venom, FS2, that differs from calciseptine by only two amino acid residues (75). Action potentials and L-type $Ca^{2+}$ currents in aortic smooth musle cells from the A7r5 cell line were potently blocked by calciseptine, although T- and L-type $Ca^{2+}$ currents in neuroblastoma cells and dorsal root ganglion cells were resistant to the toxin. Since calciseptine (1 $\mu$M) does not significantly affect contraction and T- and L-type channels in skeletal muscle cells, it is possible that calciseptine is a specific blocker of the $\alpha_{1C}$ channel of the L-type.

Calcicludine is another 60-amino acid mamba toxin, isolated from the venom of *Deudroaspis angusticeps* (76). This toxin is unique in that it blocks all major types of $Ca^{2+}$ channels except that of skeletal muscle with very high affinity ($K_d = 15$ pM), and its binding does not influence DHP or $\omega$-CgTx-GVIA binding.

### 3. Selectivity of peptide toxins in blockade of $Ca^{2+}$ channel types: Its utility for dissecting contribution of $Ca^{2+}$ channel types in biological processes

Consistent with the variability in their structure, the peptide toxins are heterologous in blocking behavior of $Ca^{2+}$ channel types (Table 1). $\omega$-CgTx-GVIA and $\omega$-Aga-IVA ($\omega$-Aga-IVB) at submicromolar concentrations are highly selective in the blockade of N- and P/Q-types, respectively (17, 60, 77, 78). Other toxins such as $\omega$-Aga-IIIA, $\omega$-CgTx-MVIIC and $\omega$-GsTx-SIA are less stringent in blocking specificity of $Ca^{2+}$ channel types, inhibiting more than a single type of $Ca^{2+}$ channel (71, 72, 79, 80). Mechanisms of action may be also variable among toxins (80, 81). Since the N-type current recovered quickly on washout when $\omega$-CgTx-GVIA was applied after block by $\omega$-CgTx-MVIIC, the two toxins may bind to the same or overlapping sites. However, addition of $\omega$-CgTx-MVIIC after block of P-type channels by $\omega$-Aga-IVA remained blocked after trains of depolarizing pulses to knock-off the toxin, which suggests distinct, simultaneous accessible binding sites for $\omega$-CgTx-MVIIC and $\omega$-Aga-IVA on P-type channels. Speculation by McDough et al. (80) was that the $\omega$-CgTx block is due to pore occlusion, but $\omega$-Aga-IVA inhibition is affected by shifting the gating toward unphysiologically positive voltages.

The peptide blockers alone and in combination have served to dissect the contribution of each type of $Ca^{2+}$ channel in various biological activities, especially neurotransmitter release (8, 54, 82–89). Most interestingly, the experiments have revealed the variable extent of contributions by different $Ca^{2+}$ channels in triggering neurotransmission, depending on the types of neuronal cells and synapses formed by them. Thus, it is quite logical that differential responses are induced by administration of different toxins.

### C. Inorganic blockers

Under physiological conditions, $Ca^{2+}$ channels must be highly selective in allowing large $Ca^{2+}$ fluxes to the exclusion of monovalent ions such as $Na^+$ and $K^+$, which underlies the most important part of the functional definition of $Ca^{2+}$ channels. Although $Ca^{2+}$ channels are even more permeable to some non-physiological charge carriers such as $Ba^{2+}$ and $Sr^{2+}$ (90, 91) and organic molecules with relatively high molecular weight (92), many others including transition metals $Ni^{2+}$, $Cd^{2+}$, $Co^{2+}$ and $Mn^{2+}$ effectively block $Ca^{2+}$ channel currents. Lanthanum ion ($La^{3+}$) is the most potent of this group of blockers (93, 94), as observed in the sequence of blocking effectiveness: $La^{3+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Mg^{2+}$ in barnacle muscle. Relative sensitivity to block by specific divalent ions is the important property that distinguishes HVA from LVA $Ca^{2+}$ channels (5, 49, 94–96). HVA channels are inhibited by submicromolar concentrations of $Cd^{2+}$ but are resistant to this concentration range of $Ni^{2+}$, whereas LVA channels have similar sensitivities to $Cd^{2+}$ and $Ni^{2+}$. $Mg^{2-}$ is the physiologically most abundant free divalent cation that blocks $Ca^{2+}$ channels, although the $Mg^{2+}$ effect is still controversial because of a lack of relief from the block by a hyperpolarizing prepulse (97, 98). $Mg^{2+}$, in the form of magnesium sulfate, is a clinically important drug: it is the primary choice to treat eclampsia (hypertension during pregnancy with convulsions). Though it has not been determined what the exact $Mg^{2+}$ concentration of $Ca^{2+}$ channel blockade is to the diverse actions of hypermagnesemia at its pharmacologically active concentration range (3–4 mM), experimental evidence points towards significant channel blockade that is antagonized by hypercalcemia. Moreover, hypermagnesemia has been shown to enhance the antihypertensive effects of DHPs, an effect utilized in the treatment of preeclampsia (99). It has been reported that millimolar internal $Mg^{2+}$ can block the inward L-type $Ca^{2+}$ or $Ba^{2+}$ currents at both the whole-cell level (97, 100) and unitary level (101) and N- and P-type channels (102). Other ubiquitous metals such as $Pb^{2+}$, $Zn^{2+}$ and $Al^{3+}$ also exert a blocking effect on $Ca^{2+}$ channels in dorsal root ganglion cells (103). These ions altogether constitute a group designated as "inorganic blockers".

Examination, for more than a quarter of century, of

the action of inorganic cations on $Ca^{2+}$ channels show that there is not a clearcut dichotomy between "pore blockers" and "permeants" (104). Analysis of the concentration dependence of permeation and block with mixtures of ions suggests that permeants and blocking ions compete for common binding sites on the channel (93, 104). The physiological permeant $Ca^{2+}$ can inhibit both the divalent and monovalent current carried by the $Ca^{2+}$ channel (104–110), which, in the absence of $Ca^{2+}$, operates as a non-selective cation channel permeable to large organic molecules such as 1,4-diaminobutane ($DAB^{2+}$) and tetramethylammonium ($TMA^{+}$) with a diameter of about 6 Å (92). Furthermore, in certain cells, some of the inorganic blockers are slightly or even very permeant (110, 111). Presumably "blocking ions" are ones that move so slowly in the pore that they prevent more mobile ions from accessing the inner pore sites (1). It is possible that subtle differences in the interaction of cations with the chemical groups of the amino acids constituting the pore is sufficient to explain why a particular bound ion can be a blocking agent in one type of $Ca^{2-}$ channel and a permeator in another (101, 112, 113).

## IV. Molecular biology of voltage-dependent $Ca^{2+}$ channels

Recent results indicate that on the basis of functional criteria it may not be possible to assign unequivocally a measured calcium current to the currently defined T, N, L and P categories and that currents of a particular pharmacological specificity may vary considerably in their biophysical properties (17–23). In addition to the four $Ca^{2-}$ channel types, two additional HVA $Ca^{2+}$ channels designated the R- and Q-type were distinguished in cerebellar granule cells (114) or in heterologous expression systems (115–119). Each type, that is now mainly distinguished using pharmacological tools, also displays a wide range of biophysical divergence. The N-type channel, distinguished from the other types by a combination of discriminatory parameters including activation at high membrane potentials, insensitivity to DHPs and susceptibility to irreversible blockade of $\omega$-CgTx-GVIA (3–5, 7), has been studied extensively to reveal complexity in its biophysical properties. They, in fact, show wide inactivation properties, although only the decaying component was originally assigned to N-type channels (3–5, 18, 20, 24, 120, 121). Inactivating and non-inactivating components are present in both the unitary and the macroscopic N-type currents, the former component being more suppressed by positive holding potentials (17, 77). Unitary N-type current, recorded in a cell-attached mode, shows multiple patterns of opening and current amplitudes (17, 122). The N-type current is modulated by neurotransmitters via G-proteins and a wide variety of second messenger mechanisms, which is restored by short strong depolarization pulses (123–130). To explain these functional fluctuations of N-type channels in native neuronal cells, different models have been employed (19, 24, 123–130). However, in general, these models have been developed, from functional data, based upon an assumption that contamination of types other than N is excluded during the measurements. Little direct information concerning the molecular basis of N-type channel diversity has been available. Thus, it has been critical to establish in detail the electrophysiological and pharmacological properties for $Ca^{2+}$ channels of known molecular identity using recombinant expression systems.

### A. Molecular diversity of the $Ca^{2+}$ channel $\alpha_1$-subunit

Over the last decade, there have been rapid advances in biochemical and molecular biological approaches to $Ca^{2+}$ channels, which have proven to be powerful methods to understand the functional diversity of voltage-dependent $Ca^{2+}$ channels.

In skeletal muscle, the L-type $Ca^{2+}$ channel is expressed as a complex of the $\alpha_1$-subunit, which is both the essential channel moiety, and the receptor for DHP and other $Ca^{2+}$ channel antagonists, and the auxiliary subunits $\alpha_2/\delta$, $\beta$ and $\gamma$ (131, 132). It is now accepted that L-types in

**Table 2.** Molecular classification of $Ca^{2+}$ channel $\alpha_1$-subunits

| Nomenclature | Tissue distribution | Functional type | Selective blockers |
|---|---|---|---|
| $\alpha_{1S}$ (Sk) | skeletal muscle | L-type | DHP |
| $\alpha_{1C}$ (C) | ubiquitous (heart, brain, aorta, lung, fibroblast) | L-type | DHP |
| $\alpha_{1D}$ (BIV) | brain, endocrine tissues | L-type | DHP |
| $\alpha_{1A}$ (BI) | brain, neuronal cell, heart | Q (P?)-type | $\omega$-Aga-IVA (<100 nM)<br>$\omega$-CgTx-MVIIC (>100 nM) |
| $\alpha_{1B}$ (BIII) | brain, neuronal cells | N-tyype | $\omega$-CgTx-GVIA (<100 nM) |
| $\alpha_{1E}$ (BII) | brain, neuronal cells | R and O-types | $Ni^{2+}$ (<100 $\mu$M) |

other tissues (133–135), as well as other types in neurons (136–144), exist as a similar but distinct complex of subunits. N-type channels composed of $\alpha_1$-, $\alpha_2$-, $\beta$-subunits are associated with the Go $\alpha$-subunit, a 95- or 110-kDa protein, as well as syntaxin and synaptotagmin, that may be involved in neurotransmitter release (139, 142–145).

There is significant structural diversity among isoforms of each subunit, arising from multiple genes and alternative splicing. Recent evidence suggests that $\alpha_1$-subunits are encoded by at least six distinct genes that comprise a gene family (see Table 2) (77, 146, 147). A phylogenetic tree, constructed by the neighbor-joining method to establish possible evolutionary relationships among $\alpha_1$-subunits, indicates that the $\alpha_1$-subunits can be classified into two subfamilies (77). One is the $\alpha_1$-subunit subfamily consisting of $\alpha_{1S}$, $\alpha_{1C}$ and $\alpha_{1D}$. The second is the subfamily consisting of $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1E}$ (BI, BIII and BII), which are predominantly or exclusively expressed in neuronal tissues. These two subfamilies probably diverged from a common ancestor, although it is unclear when the divergence occurred. It is probable that each of the subfamilies further diverged at about the same time, creating the six currently known members of the $Ca^{2+}$ channel family. The phylogenetic tree also indicates that the relationship between $\alpha_{1B}$ and $\alpha_{1A}$ is closer than those between $\alpha_{1B}$ and $\alpha_{1E}$ and between $\alpha_{1A}$ and $\alpha_{1E}$. All the $\alpha_1$-subunits share general structural features of voltage-dependent channels, particularly in the transmembrane regions (77). The $\alpha_1$-subunits contain four repeating units of homology and each repeat has one positively charged segment (S4), which may represent a voltage sensing region (148, 149), and five hydrophobic segments (S1, S2, S3, S5 and S6) (Fig. 2). The glutamic acid residues in the P ("pore") region (or SS1-SS2 segment), which is critical for ion selectivity of $Ca^{2+}$ channels (94, 150–154), are conserved among the $\alpha_1$-subunits (see below in the section V.C.3.).

### 1. L-Type $\alpha_1$-subunits

At the initial stage of the molecular approach, iden-

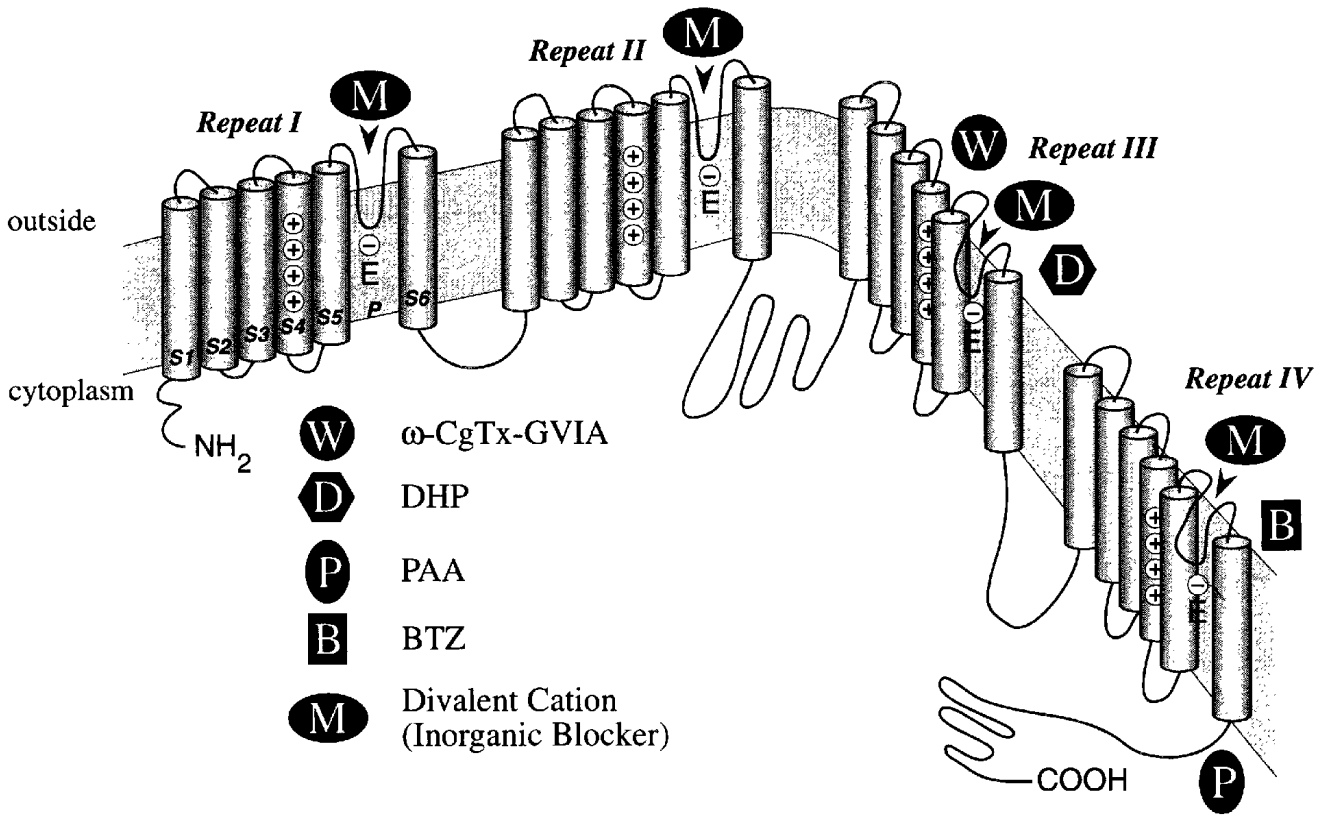



**Fig. 2.** Structural features of the drug receptor $Ca^{2+}$ channel $\alpha_1$-subunit and the interaction sites of various types of blockers.

tification of $Ca^{2+}$ channels was more directed towards the L-type isoforms. The skeletal muscle DHP receptor $\alpha_{1S}$ cDNA was the first cloned of all the $\alpha_1$-subunits (155, 156). A prominent transcript of $\alpha_{1S}$ was detected only in a RNA preparation from skeletal muscle by Northern blot analysis (156). However, successful isolation of this cDNA class from the brain, pancreatic $\beta$-cell-derived hamster insulin-secreting tumor (HIT) cells and ovary implies that this class cannot be considered as an exclusive skeletal muscle gene (157); $\alpha_{1S}$ is expressed in a developmentally regulated fashion, being induced upon myogenic differentiation (158, 159). Interestingly, $\alpha_{1S}$ cDNAs encoding a two-motif isoform have been isolated (160). Whereas $\alpha_{1S}$-specific cRNA has so far failed to direct L-type channel activity in oocytes (T. Tanabe and Y. Mori, unpublished results), skeletal muscle myotubes from mice with muscular dysgenesis (*mdg*) is a useful expression system for various classes of calcium channels; *mdg* is a fatal autosomal recessive mutation (161) that is expressed in skeletal muscle as a failure of E-C coupling (162) and the absence of the slow DHP-sensitive L-type $Ca^{2+}$ current (163), due to alteration of the structural gene for the $\alpha_{1S}$-subunit (164). Both functional defects of dysgenic myotubes were restored by microinjection of $\alpha_{1S}$ cDNA. The $\alpha_{1S}$-subunit alone directed stable and functional expression of skeletal muscle L-type channel in murine L-cells (165). Coexpression of the $\beta_{1a}$-subunit accelerated activation kinetics and increased drug binding sites, but did not increase the current amplitude (166, 167).

cDNAs encoding the ubiquitously expressed $Ca^{2+}$ channel subunit $\alpha_{1C}$ have been isolated from various tissues including heart (168), lung (169), aorta (170), brain (171), skin fibroblast cell line (172), ovarian cells and hamster pancreatic $\beta$-cell derived HIT cells (157). mRNA expression of this class in early skeletal muscle myotubes, intestine, stomach, spinal cord, pituitary, adrenal gland, liver, kidney, uterus, testes and spleen has been also shown by blotting studies (157, 170, 173, 174). Numerous isoforms of the $\alpha_{1C}$ subunit are generated by alternative splicing at different sites (157, 169–172). The variability within IVS3 is generated by a developmentally regulated, mutually exclusive splicing mechanism (175). In *Xenopus* oocytes, cRNA derived from the heart $\alpha_{1C}$ cDNA directed the formation of a functional DHP-sensitive $Ca^{2+}$ channel current (168). The $Ba^{2+}$ current carried by the channel was high voltage-activated, increased several times in amplitude by the DHP agonist (−)-(*S*)-Bay K 8644 and virtually abolished by the DHP antagonist nifedipine or $Cd^{2+}$. Electrophysiological properties of a splice variant of $\alpha_{1C}$ from lung was similar to those from heart expressed in oocytes (169). One pharmacologically interesting difference, however, did emerge from the comparison of lung and heart $\alpha_{1C}$ molecules, which differ in their amino (N)-terminus, IS6 and IVS3. Expression of the lung clone induced currents that were more sensitive to DHP antagonists than the cardiac currents (176), providing a potential explanation for the long known increased DHP sensitivity of smooth muscle $Ca^{2+}$ currents (another component of this difference in DHP sensitivity is due to the more depolarized "resting" potential of smooth muscle cells). Furthermore, analyses of the importance of the alternatively spliced region in repeat IIIS2 of the human $\alpha_{1C}$-subunit (177) indicate that IIIS2 is not part of the DHP binding site, but that two hydrophobic residues are important regulators of the voltage dependence of the DHP effects. Expression studies showed that the $\alpha_{1C}$-subunit alone is sufficient to exhibit L-type $Ca^{2+}$ channel activity. However, co-injection of the skeletal muscle $\alpha_2$-subunit enhanced the peak inward current amplitude about three times. The increasing effect on the activity of the $\alpha_{1C}$-channel was observed also for coexpression of $\beta_{1a}$ from skeletal muscle (178–180) and brain and heart $\beta_2$ (181, 182), heart $\beta_3$ (182), brain $\beta_4$ (183), and the $\gamma$-subunit (178, 179). In Chinese hamster ovary (CHO) cells, $\alpha_{1C}$ cDNA from smooth muscle alone was sufficient for stable expression of functional calcium channels (184). The single channel conductance was 26 pS with 80 mM $Ba^{2+}$ as the charge carrier, corresponding to 24.6 pS in vascular smooth muscle. The $\alpha_{1C}$ channel has also been successfully expressed in the other cell lines such as mouse L-cells (185), human embryonic kidney (HEK) 293 cells (186) and baby hamster kidney (BHK) cells (187). The cardiac L-type channel, which activates more rapidly than the skeletal muscle $Ca^{2+}$ channel, was also expressed in myotubes from *mdg* mice by nuclear microinjection of the $\alpha_{1C}$ cDNA from heart (188). E-C coupling restored by injection of the $\alpha_{1S}$ cDNA did not require entry of extracellular $Ca^{2+}$, in contrast to that restored by expression of $\alpha_{1C}$. Thus, the behaviors of the expressed $Ca^{2+}$ channels mirror the physiological situation in skeletal and cardiac muscle.

The neuroendocrine type $\alpha_{1D}$ (BIV)-subunit cDNAs were isolated from brain (189), human neuroblastoma IMR32 cell lines (190), ovarian cells (157), pancreatic islets (191) and hamster pancreatic $\beta$ cell-derived HIT cells (157). Expression of $\alpha_{1D}$ in pancreatic $\beta$ cells (191) and in the central nervous system (192) was shown by *in situ* hybridization. Multiple $\alpha_{1D}$ isoforms are generated by alternative splicing (157, 190). The recombinant expression of $\alpha_{1D}$ directed DHP-sensitive L-type activity was reversibly blocked by $\omega$-CgTx-GVIA in *Xenopus* oocytes (190). This observation, together with the distribution of both $\alpha_{1D}$ and $\alpha_{1C}$ in neurons, may imply that the $\alpha_{1D}$ channel and the $\alpha_{1C}$ channel represent the neuronal L-type that was weakly and reversibly inhibited by $\omega$-CgTx-GVIA (18) and the neuronal L-type that was insensitive to $\omega$-

CgTx-GVIA (17), respectively. Coexpression of the neuronal splice variant $\beta_{1b}$ appears to be necessary for the functional expression of the $\alpha_{1D}$ channel, and the neuronal variant of the $\alpha_2$-subunit plays an accessory role that potentiates the $Ca^{2+}$ channel activity (190).

**2. Neuronal non-L-type $Ca^{2+}$ channel $\alpha_1$-subunits**

The most recent research efforts have been in general devoted to the $\alpha_1$-subunits of $Ca^{2+}$ channels that mediate specific functions in neurons. The $\alpha_{1A}$ (BI)-subunit cDNA was the first cloned non-L-type cDNA that is distributed widely in the brain and is particularly abundant in the cerebellum (44, 193). The presence of multiple isoforms of this class, which are generated by alternative RNA splicing, has been reported (44). Blotting analysis of RNA from the cerebella of mutant mice with different types of cerebellar degeneration and *in situ* hybridization suggested that this channel is abundantly expressed in Purkinje cells, where the P-type is a major component of the whole-cell $Ca^{2+}$ current and granule cells. This class is also expressed in the pituitary (193) and weakly expressed in the heart (44, 193). Heterologous expression of this cDNA in *Xenopus* oocytes produced HVA $Ca^{2+}$ channel activity that was insensitive to nifedipine, $\omega$-CgTx-GVIA and $Ni^{2+}$, but inhibited moderately by Bay K 8644 and strongly inhibited by the funnel web spider venom and low concentrations of $Cd^{2+}$ with a half-blocking concentration of 0.5 $\mu$M. The single channel slope conductance of the $\alpha_{1A}$ channel was 16 pS with 110 mM $Ba^{2+}$ as the charge carrier. Oocytes injected with only the $\alpha_{1A}$ cRNA showed only small inward $Ba^{2+}$ currents. However, when the $\alpha_2$-subunit and the skeletal muscle $\beta_{1a}$-subunit were coexpressed, the $Ba^{2+}$ current increased by two orders of magnitude. Coexpression of the $\alpha_2$- or the $\beta_{1a}$-subunit alone increased the current to a much smaller extent, the latter being more effective than the former, whereas coexpression of the $\gamma$-subunit exerted no significant effect. While the properties of the cloned $\alpha_{1A}$ as well as its tissue distribution suggest that the $\alpha_{1A}$ channel represents the P-type channel, there are differences particularly in the low sensitivity of the expressed $\alpha_{1A}$ channel to block by $\omega$-Aga-IVA compared to the native P-type channel in Purkinje cells (60). In fact, the $\alpha_{1A}$ mimics the "Q-type" channel, inhibited partially by $\omega$-Aga-IVA, in cerebellar granule cells (114).

The $\alpha_{1B}$ (BIII) cDNAs were cloned from brain (77, 194) and human neuroblastoma IMR32 cell line (195). Two isoforms, that differ at the carboxy (C)-terminal region, are generated by alternative RNA splicing (195). Polyclonal antiserum generated against a peptide from the $\alpha_{1B}$ sequence selectively immunoprecipitates high-affinity $\omega$-CgTx-GVIA binding sites from forebrain membranes (194). The $\alpha_{1B}$ mRNA is present in all the regions of the central nervous system, including the spinal cord (77, 194, 195), calcitonin-secreting C-cells, and undifferentiated and differentiated rat pheochromocytoma PC-12 cells (194). In many neurons, $\omega$-CgTx-GVIA blocks both inactivating HVA $Ca^{2+}$ current and a part of the sustained HVA $Ca^{2+}$ current that is insensitive to DHP (17, 18, 20, 23, 24), contrary to the initial definition (4, 5), where the inactivating and sustained components of HVA $Ca^{2+}$ current were simply assigned to N-type and L-type channels, respectively. This sensitivity to $\omega$-CgTx-GVIA suggests the existence of a non-inactivating component of N current, but did not establish whether the sustained and inactivating components arose from the identical channel molecule. The recombinant $\alpha_{1B}$ current was assessed by microinjecting the $\alpha_{1B}$ cDNA into nuclei of myotubes from *mdg* mutant mice (77). This expressed channel gave rise to both inactivating and non-inactivating $Ca^{2+}$ currents that were inhibited by $\omega$-CgTx-GVIA, which supports the concept that L- and N-types cannot be distinguished on the basis of inactivation kinetics but through differences in pharmacological sensitivity. Additionally, steady state inactivation properties cannot be a useful criteria of distinguishing N-type channels from other types since a 1-sec prepulse to $-20$ mV had a comparatively small effect on the maintained component of current through $\alpha_{1B}$ channels while almost completely inactivating the transient component. In other systems such as *Xenopus* oocytes (118, 196, 197) and HEK cells (195), $\alpha_{1B}$ directed the recombinant expression of N-type calcium channel activity transiently.

The spatial distribution of the $\alpha_{1E}$ (BII)-subunit, in the brain is different from that of other members of the neuronal $\alpha_{1A}/\alpha_{1B}/\alpha_{1E}$ subfamily, being abundant in the cerebral cortex, hippocampus and corpus striatum (117–119, 198–200). The $\alpha_{1E}$-subunit expressed in oocytes was a HVA $Ca^{2+}$ channel pharmacologically featured by its unique sensitivity to the inorganic blocker $Ni^{2+}$ (116–119, 199). The decaying component of the $\alpha_{1E}$ current showed high sensitivity to $Ni^{2+}$ similar to that of the LVA T-type and the HVA R-type channel in cerebellar granule cells, whereas the sustained component was relatively resistant to $Ni^{2+}$ as are the other HVA $Ca^{2+}$ channels. In addition, DHPs, $\omega$-CgTx-GVIA and $\omega$-Aga-IVA, which have been used to discriminate L-, N- and P-types, did not affect the $\alpha_{1E}$ current. Thus, the $\alpha_{1E}$ channel forms a novel functional category distinct from the four conventional categories of $Ca^{2+}$ channel.

**B. Molecular diversity of auxiliary subunits**

Molecular diversity has also been demonstrated for the other subunits. Cloning and sequencing of the cDNA of the $\alpha_2$-subunit isolated from skeletal muscle revealed that

the $\alpha_2$-subunit is a heavily glycosylated hydrophilic protein without homology to other known protein sequences (156). The $\alpha_2$-subunit contains three hydrophobic segments, which may constitute transmembrane domains of the protein. Northern blot analysis and immunoblot analysis suggests that the $\alpha_2$-subunit is present in a variety of tissues (156), induced upon myogenic differentiation (158, 159). The lack of a significant difference between the patterns of DNA blot analysis of genomic DNA under conditions of high and those of low stringency (156) suggests that significant homology exists between the skeletal muscle $\alpha_2$-subunit and the $\alpha_2$-subunits expressed in the other tissues. Indeed, the cDNA of the $\alpha_2$-subunit isolated from brain encoded a splice variant of the skeletal muscle $\alpha_2$-subunit (190). N-terminal sequence analysis of the $\delta$-subunits revealed identical sequences that match the C-terminal portion of the predicted amino-acid sequence of the $\alpha_2$-subunit (201, 202), supporting a model of the $\alpha_2$-subunit in which the propeptide processed into two chains that remained attached through disulfide linkages.

The existence, to date, of four distinct $\beta$-subunit genes has been reported (designated as $\beta_1$, $\beta_2$, $\beta_3$ and $\beta_4$). An analysis of the amino acid sequence of the $\beta$-subunit reveals the absence of a typical membrane region and weak homology to some isoforms of actin, keratin, glial fibrillary acidic protein, myosin and tropomyosin and the presence of several potential cyclic AMP-dependent phosphorylation sites (203), supporting biochemical analyses of the subunit. Subunits $\beta_1$, $\beta_2$, $\beta_3$ and $\beta_4$ are all expressed in the brain (181–183, 203, 204). As with the other subunits, the cDNA encoding the $\beta$-subunit ($\beta_{1a}$) was first cloned from skeletal muscle (203), and later its splice variant ($\beta_{1b}$) from brain was cloned (190, 204). The $\beta_2$- and $\beta_3$-subunit cDNAs were isolated from heart and brain (181, 182). Northern blot analysis revealed the expression of $\beta_2$ not only in the heart and brain but also in the aorta, lung, trachea, and two insulin-secreting tumor cell lines HIT (hamster) and RIN5mF (rat) (181, 182) and that of the class $\beta_3$ in the heart, skeletal muscle, brain, aorta, trachea and lung (182). The $\beta_4$-subunit is most abandunt in the brain, particularly in the cerebellum (183).

The $\gamma$-subunit and syntaxin are more restricted in tissue distribution than the $\alpha_1$-, $\alpha_2$- and $\beta$-subunits. The primary structure of the $\gamma$-subunit deduced by sequencing of the cDNAs cloned from skeletal muscle indicates that the $\gamma$-subunit is a glycosylated protein with four putative transmembrane domains (205, 206). No amino-acid sequences of other known proteins showed significant homology with the sequence of this subunit. Expression of the $\gamma$-subunit mRNA was also detected in the lung but not in neuronal tissues (205). Isoforms of syntaxin are widely expressed in the nervous system (142, 145, 207–209).

Most recently, interaction of the $\alpha_1$-subunits with the auxiliary subunits have been characterized. The interaction sites of the $\beta$-subunit and syntaxin mapped in the primary structures of the $\alpha_1$-subunits are intracellular linker regions I–II and II–III, respectively (210–212). In the $\beta$-subunits, a small N-terminal portion of the C-terminal half was shown to be responsible for the $\alpha_1$-$\beta$ interaction (213, 214). For the $\alpha_1$-$\alpha_2/\delta$ interaction, less structural information is available. It has been suggested that the interaction is through transmembrane domains and both the $\alpha_2$- and $\delta$-subunits are important for the interaction (215). Similarly, glycosylation of the $\alpha_2/\delta$-subunit is essential for proper interaction with the $\alpha_1$-subunit. This interaction may be of particular importance, since it has been shown that the $\alpha_2/\delta$-subunit cosegregates with the malignant hyperthermia phenotype in certain families and one may assume that if the largely extracellular $\alpha_2/\delta$-subunit is involved in a disease of skeletal muscle $Ca^{2+}$ release (216), its effect ought to be transduced through its interaction with the $\alpha_1$-subunit.

## V. Molecular and structural identification of binding sites for $Ca^{2+}$ channel blockers

### A. Electrophysiological identification of drug-$Ca^{2+}$ channel interaction: Use of permanently charged derivatives

All of the three major $Ca^{2+}$ antagonists have a secondary or tertiary amino group. However, because the amino groups are in equilibrium of neutral as well as charged forms at physiological pH and the uncharged form can easily permeate the plasma membrane to accesss residues of the channel proteins on the other side of the membrane, the original forms of the drugs could not be used to determine the sidedness of the action relative to the plasma membrane. In other words, these drugs were not sufficient to rule out or prove intracellular or extracellular access to the drug binding site. To circumvent this problem, drug derivatives modified to permanently ionized quaternary forms with restricted membrane accessibility have been used to characterize the sidedness of their action.

It was originally implied by Oya et al. that a methoxy verapamil, (−)D600, acts on the L-type channels from the outside in isolated vascular and ileal smooth muscle cells of the rabbit (217). This hypothesis was reinvestigated by comparing the effects of external application and intracellular application of D890, a quaternary form of PAA D600, through a patch-recording pipette on L-type channels in heart cells (218) and in smooth muscle cells (219). The results of the studies indicate that the quaternary derivative blocked $Ca^{2+}$ channel currents only

from the intracellular membrane surface. The idea is somewhat consistent with the dependence of the D600 block on membrane potential depolarization and repetitive depolarizing pulses and the resemblance of the PAA action with that of tertiary or quaternary local anesthetics (1).

DHP derivatives, such as PN200-110, nitrendipine, and nifedipine, that have pKa values less than 3.5 (220) are neutral molecules at physiological pH so that they are also accessible to the binding site from either side of the lipid bilayer. Kass and Arena (221) used amlodipine, a tertiary DHP (pKa of 8.6), that is 94% ionized at physiological pH, to probe the DHP binding site. Under conditions (pH) that favor neutral drug molecules, amlodipine block resembled those of other neutral DHP drugs: the block was more pronounced at depolarizing voltages, recovery from the block was complete at hyperpolarized voltages, but repetitive pulsing did not enhance the block. When the drug was ionized, the time course of the enhancing effect of depolarizing voltages was slow and sped up by repetitive pulses that opened the channels. Furthermore, recovery from the block by the ionized form was very slow and incomplete, and it became rapid when the pH was changed from 7.4 to 10.0. These results resembled those obtained for $Na^+$ channels blocked by local anesthetics that interact with the intracellularly located binding site. However, an examination, carried out by the same group, of a fully charged quaternary compound SDZ 207-180 localized the DHP interaction site on the extracellular side, in contrast to quaternary PAA D890 (222, 223). Extracellular application of SDZ 207-180 caused voltage-dependent block of $Ca^{2+}$ channel currents, whereas the drug as well as amlodipine was ineffective when applied intracellularly. Under conditions in which $Ca^{2+}$ is the permeant ion, block by ionized amlodipine and SDZ 207-108 develops at a slower rate than under conditions where extracellular $Ca^{2+}$ concentration is buffered and monovalent cations carry the charge. Block by the neutral nisolidipine is not affected by chelating extracellular $Ca^{2+}$.

A permanently charged benzazepine, SQ 32.428, which is a competitive inhibitor of dilitiazem (BZT) binding, was used to localize the approximate binding site of BZT/benzazepine antagonists (224). Upon extracellular application, the compound was able to completely block $Ca^{2+}$ currents, whereas intracellular application of SQ 32.428 did not change the time course of $Ca^{2+}$ currents compared to control run-down without application of the drug. These results suggest an extracellular location of the benzazepine/BZT interaction site on the $Ca^{2+}$ channel complex.

FPL 64176, a benzoyl pyrrole compound, has been suspected to act on or near the extracellular face of the L-type channel (39). Rampe and Lacerda showed that FPL 64176 increased current amplitude only after the drug was applied to the outside of the cell, even though the drug was present in the patch pipette throughout the measurement.

The experiments using permanently charged quaternary derivatives of $Ca^{2+}$ antagonists has introduced a view that the binding sites for DHPs, BTZs and PAAs are physically separate entities. In the next section, we will expand the view into a concept that the three major drug groups interact with each other from distinct sites on the $\alpha_1$-subunit.

### B. Biochemical characterization of drug-$Ca^{2+}$ channel interaction: Photoaffinity labeling of $Ca^{2+}$ channels

Earlier experimental observations derived from photoaffinity-labeling studies suggested that regions of the $\alpha_1$-subunit contain the binding sites for all of the three major classes of $Ca^{2+}$ antagonists (131, 132). The binding sites within the skeletal muscle $\alpha_1$-subunit polypeptide have been identified using photoreactive drug labeling and purification. The drugs bind to high-affinity receptor sites, and upon photoactivation, they covalently label a region that is in or in close proximity to the high-affinity binding site on the polypeptide. Specifically labeled $\alpha_1$-subunit preparations were then subjected to limited proteolysis with various proteases and then the mapping of the labeled region with a panel of antibodies specific to the $\alpha_1$-subunit polypeptide subfragments.

A photoactive verapamil, LU49888, was utilized to determine the binding site for PAAs on the rabbit skeletal muscle $\alpha_{1S}$-subunit solubilized and partially purified by affinity chromatography. The covalent label for PAA was localized on a 42-amino acid (Glu-1,349−Trp-1,391) segment that encompasses transmembrane segment S6 in motif IV and short adjacent sequences from the intracellular tail of the $\alpha_{1S}$-subunit (225, 226). Similar photoaffinity-labeling strategies were employed to reveal two labeling sites on the $\alpha_{1S}$-subunit for DHPs such as the arylazide azidopine, the diazirine diazepine and the bezofurazene (+)-PN 200-110 (isradipine) (226−228). The sites comprise part of the transmembrane segment S6 and the adjacent extracellular loop in motif III as the primary site and the corresponding region in motif IV as the "secondary or peripheral" site. Somewhat different conclusions were reached when photoaffinity labeling of skeletal muscle membrane preparations was followed by purification and trypsin digestion of the labeled components (229). The specific label was found in the extracellular loops between S5 and S6 segments of motifs I, III and IV, but not in the intracellular C-terminal region adjacent to IVS6. However, the original report by Regulla et

al., located the DHP binding in the opposite intracellular tail in the C-terminal region (230). It is possible that this discrepancy derives from differences in the experimental procedures (see V.C.1. below). Azidobutyryl clentiazem, a photoreactive analog of diltiazem, specifically labeled the S5–S6 loop of motif IV (231). Thus, the labelling pattern is in accordance with the previous observation that the permanently charged quaternary PAA D890 inhibited the $Ca^{2+}$ channel current only from the intracellular side (see the section above), and the quaternary DHP SDZ 207-180 as well as the benzazepine SQ 32.428 inhibited it only from the extracellular side.

### C. Molecular biological characterization of drug-$Ca^{2+}$ channel interaction: Studies with experimental $Ca^{2+}$ channel mutants

#### 1. Organic blockers

The recent introduction of molecular biological techniques to the field enabled investigators to determine the complete primary structure of pharmacological targets but also encouraged speculation concerning the significance of particular regions or even particular amino acid residues through examination of functional alteration induced by artificial substitution in the amino acid sequence. For studying structure-function relationships of different ion channels, in fact, the combination of cDNA expression and electrophysiological analysis have provided a powerful tool. Structural determinants of E-C coupling (232) and channel activation kinetics (233) have been located in the primary structure of the DHP-sensitive L-type $Ca^{2+}$ channel $\alpha_1$ using chimeras between skeletal muscle $\alpha_{1S}$ and cardiac $\alpha_{1C}$ expressed in *mdg* myotubes. Furthermore, it has been demonstrated that the glutamic acid residues in the pore-forming "P" region of the four repeats, equivalently positioned in the aligned sequences, play different roles in the selective permeability of $Ca^{2+}$ channels (151–154), in agreement with the data and model demonstrated for $Na^+$ channels and $K^+$ channels (150, 234–239). The advantage of the approach is that structural evaluation is always carried out relying upon the functional channel proteins free from any conformational "destruction" caused during biochemical procedures. Furthermore, attempts to determine the DHP site using photoaffinity-labeling methods turned out to be more controversial than for other drugs, assigning DHP-binding to different regions (225–230). This inconsistency appears to derive from at least three characteristic drawbacks of the photolabelling procedure (147): i) photoreactive groups on the side chain are at a distance of 10–15 Å from the core structure of the derivatives; ii) the limited availability of antibodies that can precipitate proteolytically digested short fragments of the $\alpha_1$-subunit; iii) the labeling pattern somewhat depends on whether the photolabeled sample is a purified or crude membrane preparation.

To overcome these intrinsic problems of the photoaffinity-labeling approach, a chimeric approach was utilized (240). In a series of experiments, chimeric $\alpha_1$-subunits engineered between cardiac $\alpha_{1C}$ and brain $\alpha_{1A}$ (BI $\alpha_1$) that produce the DHP-sensitive L-type and DHP-insensitive Q-type $Ca^{2+}$ channels, respectively, were constructed and expressed in *Xenopus* oocytes to test for modulation by DHPs. A chimera, in which a region from S3 to S6 of repeat IV of the L-type $\alpha_{1C}$ was replaced with the corresponding region of the DHP-insensitive $\alpha_{1A}$, exhibited no DHP sensitivity. In another chimeric construct, which has the $\alpha_{1A}$-channel region from S5 to S6 of repeat IV, a markedly decreased sensitivity and efficacy of DHP agonists was observed, but antagonist sensitivity was fully retained. However, a chimera in which parts of the repeat III S5-S6 connecting loop and S6 segment were replaced with the corresponding region from the $\alpha_{1A}$-subunit retained sensitivity to both DHP antagonists and agonists. These studies clearly emphasize the critical involvement of the repeat IV S5-S6 loop and part of the segment S6 in the functional binding domain of DHPs (Fig. 2). Most recently, Grabner et al. have demonstrated that DHP sensitivity is transferrable to the $\alpha_{1A}$ channel using the same chimeric approach (241). A minimum stretch of the $\alpha_{1C}$ sequence sufficient for transferring DHP sensitivity includes IIIS5, IIIS6 and the connecting linker, as well as the IVS5-IVS6 linker and IVS6, covering 9.4% of the total sequence. Different regions in repeat IV are responsible for the response to DHP agonists and antagonists: critical structural determinants for agonist action of DHP are located within the transmembrane segment S6, whereas those for DHP antagonist action are in the N-terminal half of the S5-S6 linker. Taken together, using both photoaffinity-labelling and chimeric techniques, the data to date suggest that DHPs and BTZs bind to a region in close proximity to the pore lining region. Binding of the DHPs and BTZs may introduce "distortion" into the polypeptide that propagates into the pore structure, thereby reducing the probability of the permeable "open conformation" of the L-type channel.

Further attempts to obtain a more detailed map of amino acid residues that are involved in the interaction with DHPs was made by Mitterdorfer et al. (242) and Peterson and Catterall (243). It has been reported that DHP-binding is allosterically stabilized by $Ca^{2+}$ binding with a $K_d$ less than 1 $\mu$M in the DHP-sensitive L-type $Ca^{2+}$ channels (29). To examine the involvement of the high-affinity $Ca^{2+}$-binding site in the allosteric coupling of $Ca^{2+}$ binding and the DHP binding, site-directed mutations of conserved E residue, that have been shown to

form a high-affinity $Ca^{2+}$-binding site(s), and the phenylalanine (F) residue in repeat III were introduced in the pore-forming "P loop" (previously called the SS1-SS2 region) of the recombinant DHP-sensitive L-type channels. Neutralization of the $Ca^{2+}$-binding E residues, E393 in repeat I and E736 in repeat II, by substituting with Q impaired $Ca^{2+}$ stimulation of isradipine binding to the rabbit heart L-type $\alpha_{1C}$-subunit, as a consequence of acceleration of $Ca^{2+}$ dissociation (242). Replacement of the $Ca^{2+}$-binding residues E1,014 in repeat III and E1,323 in repeat IV with Q caused 11- and 35-fold decreases in sensitivity of the skeletal muscle L-type $\alpha_{1S}$ channel expressed in tsA-201 cells to the enhancement of (+)-PN200-110 binding by $Ca^{2+}$ (243). Substitution of F1,013 in repeat III of $\alpha_{1S}$ with Gly (G), as in the correponding position of DHP-insensitive $Ca^{2+}$ channels, exerted a fourfold decrease of $Ca^{2+}$ sensitivity. The results suggested that high-affinity $Ca^{2+}$ binding to the Glu residues in the P regions of repeats III and IV of $\alpha_{1S}$ stabilizes the DHP receptor site in its high-affinity state.

Sites responsible for high-affinity PAA block of L-type $\alpha_{1C}$ channels has been also mapped in its primary structure (244). Site-directed mutations Y1463A, A1467S or I1470A in repeat IV S6 6−12 times increased the $K_d$ value of (−)D888, and those of paired combinations of these mutations were even >100 times less sensitive to the drug, supporting the previous photoaffinity-labeling studies.

**2. Peptide toxins: ω-Conotoxin-GVIA**

Neurotoxins that selectively block $Na^+$, $K^+$ or $Ca^{2+}$ channels have served as useful tools to probe the diversity of the voltage-gated channel superfamily. In comparison to $Na^+$ and $K^+$ channels, little is known about the molecular mechanism of $Ca^{2+}$ channel susceptibility to blockade by groups of peptide toxins; it is unknown whether the block occurs via channel occlusion or modification of the gating apparatus. Interestingly, one may also consider peptide toxins as molecular probes to gain insight into the three-dimensional structures of ion channels. For example, charybdotoxin (a toxin of *Centruroides scorpious*) has been successfully used to map its receptor site on $Ca^{2+}$ activated as well as on *shaker* $K^+$ channels (245). This may warrant a similar approach for $Ca^{2+}$ channel inhibitory toxins.

As described above, ω-CgTx-GVIA, a selectively blocker of N-type channels, and ω-Aga-IVA/B, a selective blocker of P/Q-type, interestingly share the same topological characteristics in the three-dimensional structure, which implies a common molecular interaction for the inhibitory action of these peptide toxins on $Ca^{2+}$ channels, although several differences in modes of inhibition can be identified between ω-conotoxins and ω-agatoxins (80). These toxins are among the best studied peptide toxins in regard to their effect on a variety of neuronal processes and the mode of inhibitory action (see above). Thus, these logical and rational attempts to generalize the structural information obtained for the interaction of $Ca^{2+}$ channels with the ω-conotoxin or ω-agatoxins have revealed useful information that could be extrapolated to other peptide toxins.

Recently, structural determinants of the N-type $Ca^{2+}$ channel's susceptibility to ω-CgTx-GVIA has been described by Tsien's group (197). Chimeric $Ca^{2+}$ channels were systematically constructed from the ω-CgTx-GVIA-sensitive N-type $\alpha_{1B}$ channel and the ω-CgTx-GVIA-insensitive Q-type $\alpha_{1A}$ channel, which is unresponsive to ω-CgTx-GVIA, and the time courses of ω-CgTx-GVIA blockade among $Ca^{2+}$ channel currents produced by the chimeras were examined. The onset of the toxin inhibition was most strikingly impaired when repeat III of the N-type $\alpha_{1B}$-subunit was replaced with the corresponding region of the $\alpha_{1A}$-subunit. Further mutation studies have located amino acids that are the most critical for high-affinity ω-CgTx-GVIA inhibition, within the putative extracellular loop between S5 and P region in repeat III. Considering the previous reports locating the glutamate residue with the highest $Ca^{2+}$ affinity in the repeat III P loop (149−154) as well as the critical importance of motif III in the proton block of the channel (246, 247), the results obtained are consistent with a direct pore-blocking mechanism (Fig. 2).

**3. Inorganic blockers**

As mentioned above in Section III.C., there appears to be no real qualitative difference between "pore block" and "ion permeation". If the channel pore is endowed with a specific molecular apparatus, the so-called "selectivity filter", it may be an inevitable consequence for a channel to be susceptible to blockade by the ions that share similar chemical properties with the permeants for the high-affinity binding to the pore but can hardly leave the pore. Three general hypotheses that involve "high-affinity $Ca^{2+}$ binding sites" have been proposed to describe the interaction of blocking ions and permeants with the $Ca^{2+}$ channel pore. One model postulates the existence of two separate, high-affinity sites close to both ends of the pore (105, 109), where high-affinity binding of $Ca^{2+}$ secures high selectivity to $Ca^{2+}$ over other outnumbering monovalent cations, and the ion-ion interaction (repulsion) of two $Ca^{2+}$ molecules at the sites in the single file pore allows high $Ca^{2+}$ flux. In the second model, only one high-affinity site with a net charge of −2 e is postulated (248). Decrease in the free energy level allows the entry of a second $Ca^{2+}$ to the site, which eventually leads to the exit of the first $Ca^{2+}$. The third model is different from

the other two models. The high-affinity site is externally located outside the permeation pathway to exert an allosteric switch between selective conformation and non-selective conformation (249).

Multiple ion occupancy of the channel pore is primarily dependent on the observation of an anomalous mole fraction effect between $Ca^{2+}$ and $Ba^{2+}$ (109, 250–252), but this observation has not been fully characterized experimentally after a decade following the proposal of the hypotheses. Kuo and Hess have systematically characterized the physiochemical nature of the high-affinity binding sites and ion-ion interaction in the pore by examining the kinetics of blockade by different divalent metal cations of unitary L-type currents in rat phaeochromocytoma PC-12 cells (101, 112, 113). Regardless of which side $Ca^{2+}$ is coming from to block the monovalent $Li^+$ current, the exit rates at the same potential were always the same and in the same direction of the $Li^+$ current at the same potential. This indicates that internal and external $Ca^{2+}$ both may go to the same binding site in the pore to produce the block, suggesting that there is a single high-affinity site (or a set of such sites separated by insignificant barriers) for $Ca^{2+}$ binding. Fast on-rate for external $Ca^{2+}$, which even reached the rate of diffusion control ($10^9\,M^{-1}s^{-1}$), locates the high-affinity sites close to the external mouth of the pore. The same sites were also suggested for $Cd^{2-}$ and $Mg^{2+}$ binding in the blockade of unitary divalent ($Ca^{2+}$ and $Ba^{2+}$) and monovalent ($Na^+$ and $Li^+$) currents, respectively (96, 104–106). Furthermore, since the blocking ion ($Ca^{2+}$) always leaves the pore with the current, there should be high-affinity sites where one blocking ion and one permeant ion can be simultaneously accommodated to generate ion-ion interaction in the pore.

Structural determinants for inorganic cation blockade and ion selectivity were first identified in the neuronal $\alpha_{1A}$ channel (94, 253). In the study, the negatively charged glutamate residue (E) in each P-loop (previously called as SS1-SS2 region) (147) was replaced with a neutral glutamine residue (Q) by an in vitro mutagenesis technique, and the divalent ($Ba^{2+}$) and monovalent ($Na^+$) currents produced by the mutants in *Xenopus* oocytes were examined for their response to various inorganic blockers (Fig. 2). Sensitivity to $Cd^{2+}$ blockade of $Ba^{2+}$ currents and that to $Ca^{2+}$ blockade of $Na^+$ currents differed among mutants in the order of wt (wild type)>IV>II $\gg$I>III and wt>II>IV>I>III, respectively. The dissimilarity among mutant currents, also observed for reversal potentials in high $Ba^{2+}$ solution, indicates that four glutamates assume an asymmetrical geometry in the pore to form high-affinity binding sites for both divalent blockers and permeants. However, since the severity of mutation tends to be greater in the repeats I and III than repeats II and IV, each pair of glutamates may be located at a similar depth along the pore in the $\alpha_{1A}$ channel.

Even more systematic analysis was carried out on the DHP-sensitive L-type $\alpha_{1C}$-subunit (151–154). Not only single substitutions for E with Q in the P-loop of each repeat, but also substitutions with aspartates (D), alanines (A), and lysines (K), or even double and quadruple mutations to these residues were introduced in the $\alpha_{1C}$-subunit channel. Replacement of all the P-loop E residues with A or K abolished the micromolar divalent cation block of $Li^+$ current without revealing any other high-affinity binding sites (152–154). The major involvement of four P-region E residues in the formation of the binding site is in line with the previous prediction (254) and with the pH titration experiment (113). The disparity between the $Cd^{2+}$ sensitivity of $Li^+$ currents and that of $Ba^{2+}$ currents is much less pronounced in the mutants, supporting the idea that $Ba^{2+}$ can displace $Cd^{2+}$ through ion-ion interaction (154). The concept that the four E residues in the P-regions are arranged asymmetrically in the formation of high-affinity sites in the lumen of the channel can be also applicable for the $\alpha_{1C}$-subunit. The sensitivity to $Cd^{2+}$ blockade of $Ba^{2+}$ currents and that to $Ca^{2+}$ blockade of $Li^+$ currents produced by mutants in the order of wt>IV>II>I>III and wt>I>IV>II>III, respectively, suggesting that the relative extent of contribution of each E in the L-type $\alpha_{1C}$ was significantly different from that in the $\alpha_{1A}$ channel. The repeat IV E mutation was unusual, because, of the single E to K substitutions, only that in repeat IV carried significant $Ba^{2-}$ currents. It cannot be determined completely whether the IV E is structurally deviant from the cluster of other E residues or the role of this E is replaced by other surrounding E residues in the mutant, which is consistent with the decrease in single channel conductance observed. In contrast to other E residues, repeat I E was more sensitive to the E to D mutation than the E to Q mutation (154), which, together with the uniqueness of the E to A mutation in repeat I in exerting a strong effect on the affinity of $Ca^{2-}$ against the background of E to A mutation in the other repeats compared to the other E to A mutations, implies that the repeat I E is sterically constrained by other E residues to provide only part of the carboxylate group for $Ca^{2+}$ binding.

The E residues of the selectivity filter, in addition to their role in divalent cation binding, were suggested to be involved in the pH regulation of $Ca^{2+}$ channels (113). Protons exert their blocking effect through shifting the $Ca^{2+}$ channels from a high conductance to a lower conductance state. Recently, it has been shown that replacement of the E residues of repeat I or III with Q abolished the high conductance state (246). Alternatively, when A

substitutions were used (an amino acid with a side chain that cannot be protonated under any condition), the mutation in repeat III not only abolished the lower conductance, proton blocked state, but rendered the $Ca^{2+}$ channel conductance pH-insensitive (247), indicating that repeat III E has a preeminent position in conferring the pH block of the $Ca^{2+}$ channel. It is interesting to note that none of the point mutations introduced in S5-S6 linkers caused any desruptive effect on DHP sensitivity and voltage dependence of activation of the $\alpha_{1C}$-subunit and that the residues other than the E residues mutated so far in the S5-S6 linkers did not cause any significant effect on ion permeation and its block by inorganic cations.

Thus, the mutation experiments have established the structural basis of the "selectivity filter" constituted from the high-affinity sites for cation binding in different types of voltage-dependent $Ca^{2+}$ channels. They have also revealed that formation of "selectivity filters" by P-regions in S5-S6 linkers is a structural principle shared by different types of channels including voltage-gated $Na^+$ and $K^+$ channels (150, 234–239). The detailed chemical interaction of the ions bound to the sites is not yet known. It is possible that differences in the mobility of the ions along the sites will become one of the most important factors that determine whether the ion is a "blocker" or a "permeator".

## VI. Modulation of pharmacological sensitivity of voltage-dependent $Ca^{2+}$ channels by the auxiliary subunits

cDNA expression studies have demonstrated that the $\alpha_1$-subunit alone constitutes the channel pore and gating apparatus (44, 165, 168), and that the auxiliary subunits regulate in some way the expression and/or detailed "behavior" of the channel, exerting pronounced effects on macroscopic characteristics such as kinetics, voltage dependence of activation, and inactivation (44, 166–168, 178, 182, 190, 195) and single channel properties (255, 256). Of considerable interest and importance are the biochemical analyses that have revealed that the $\alpha_1$-subunit carries the specific receptor sites for at least three classes of organic $Ca^{2-}$ channel blockers (131, 132). If the auxiliary subunits do indeed influence multiple parameters of $Ca^{2+}$ channel function through induction of conformational changes in the $\alpha_1$ proteins, it is reasonable to presume that interaction of the $\alpha_1$-subunits with various pharmacological agents is also affected. Numerous studies have been conducted to resolve the issue whether the auxiliary subunits modulate sensitivity of $Ca^{2-}$ channels to various pharmacological agents.

Coexpression of the $\alpha_2$-subunit together with the cardiac L-type $\alpha_{1C}$ current expressed in oocytes by injection of the respective cRNAs provided the first opportunity to examine effects of auxiliary subunits on pharmacological properties of $Ca^{2+}$ channel currents (168). There was, however, no significant detection of the influence of the accessory subunits on the response to the DHP agonist Bay K 8644 or the DHP antagonist nifedipine. More extensive studies, using the same $\alpha_{1C}$ together with the $\alpha_2$- and $\beta_2$-subunits indicated that DHP sensitivity of the L-type $\alpha_{1C}$ current was not affected by the coexpression of the auxiliary subunits (181). Concentration for half-maximal stimulation ($EC_{50}$) by Bay K 8644 was approximately the same (30 nM) for both $\alpha_{1C}$ alone and $\alpha_{1C}+\beta_2$. Nor did the $\beta_3$-subunit significantly affect the block of $\alpha_{1C}$ currents by PN200-110 or mibefradil (Ro 40-5967) in CHO cells (257). The $\alpha_{1A}$ channel isoforms coexpressed together with $\alpha_2$ and/or $\beta_{1a}$ showed pharmacological properties indistinguishable from those of $\alpha_{1A}$ expressed alone (44). The combination with other $\beta$-subunit classes and splice variants failed to enhance the $\omega$-Aga-IVA sensitivity of $\alpha_{1A}$ currents to the level of native P-type channels in cerebellar Purkinje neurons (114, 115, 258, 259). Auxiliary subunits also did not affect the sensitivity of the $\alpha_{1A}$ $Ca^{2+}$ channel to $\omega$-CgTx-MVIIC, although the blocking time course of the toxin apparently differed slightly depending on the subunit composition (259). Furthermore, recent examination on $\omega$-CgTx-GVIA sensitivity of the $\alpha_{1B}$ channels expressed in *Xenopus* oocytes in different subunit combinations have indicated that the auxiliary subunits $\alpha_2$ and $\beta_{1b}$ do not exert significant effects on the $\omega$-CgTx-GVIA response of the recombinant N-type channels (ref. 196; M. Wakamori et al., unpublished results). Several binding studies support these electrophysiological observations. The $K_d$ values for binding of DHP such as that of PN200-110 binding to recombinant $\alpha_{1S}$ (166, 167) or the $\alpha_{1C}$ channel (176, 260) was unaffected by coexpression by accessory subunits. Similarly, there were no significant differences among $K_d$ values of $\omega$-CgTx-GVIA-binding to N-type channels heterologously expressed in various subunit combinations (refs. 195, 261; M. Wakamori et al., unpublished results).

Although one of the trends is that the auxiliary subunits do not affect the affinity of a variety of drugs to $Ca^{2+}$ channels, there is also some unneglectable data implying modulation of the binding affinity. Strong regulation of DHP sensitivity of the recombinant skeletal muscle $\alpha_{1S}$ current by coexpression of the $\beta_{1a}$-subunit has been demonstrated in mouse L cells (166). Unexpectedly and contrary to the DHP binding data (see above), $Ba^{2+}$ currents induced by the combination of $\alpha_{1S}$ with large excess of $\beta_{1a}$ were not responsive to the enhancement effect of Bay K 8644. The $\beta_{1a}$-subunit effect was partially reversed by the coexpression of the $\alpha_2$-subunit. A decrease in Bay K 8644 enhancement of the $\alpha_{1S}$ current amplitude paralleled with an increase in the expression level of the

$\beta_{1a}$ mRNA (262). These results raise the possibility that the L-type $\alpha_{1S}$-subunit, together with other subunits, can generate two types of $Ca^{2+}$ channel currents, a DHP-modulatedore and a DHP-resistantore, in line with the hypothetical dual functional role of the $Ca^{2+}$ channel as a voltage sensor for excitation-contraction coupling and as a $Ca^{2+}$ channel to replenish $Ca^{2+}$ in skeletal muscle (155). The difference in efficiency of potentiation by Bay K 8644 was observed among $Ca^{2+}$ channel currents produced by possible combinations of the $\alpha_{1C}$-, $\alpha_{2}$-, $\beta_{1a}$- and $\gamma$-subunits (178). Hullin et al. concluded that all the $\alpha_{1C}$ channels that also contain $\beta$-subunits are less sensitive to Bay K 8644 than the other combinations, by comparing percent increases in $Ba^{2+}$ currents by Bay K 8644 (182). However, since coexpresssion of the $\beta_{1b}$-subunit increases the Po of unitary L currents (255), this finding may be simply due to the mathematical consequence that the difference between Po values before and after the Bay K 8644 application is less pronounced for the combination $\alpha_{1C}+\beta$ than $\alpha_{1C}$ alone, if the maximum Po level in the presence of the agonist is the same for $\alpha_{1C}+\beta$ and $\alpha_{1C}$ alone. Moreover, since both $\beta$-subunits and Bay K 8644 induce a shift in the I-V curve, it is necessary to be very careful in evaluating the currents at any given test potential: the equilibrium of conformomeres is shifted, causing possible artefacts in the response to a drug that binds in a conformation-dependent manner. The $\beta_3$-subunit significantly decreased the half-maximal inhibitory concentration of the PAAs (−)-gallopamil and verapamil in CHO cells (257). Using COS cells, in which so far no functional expression of recombinant $Ca^{2+}$ channel has been reported, Mitterdorfer et al. have demonstrated that coexpression of the $\beta_{1a}$-subunit resulted in a >35-fold increase in affinity of PN200-110-binding (242).

Thus, it is still controversial whether auxiliary subunits exert any effect on the pharmacological sensitivity of $Ca^{2-}$ channels. The discrepancies may derive from the usage of multiple recombinant expression systems such as *Xenopus* oocytes, COS, CHO and HEK cells. These systems probably have different intracellular mileus that are expressed as differences in phosphorylation and other post translational modifications and interactions.

## VII. Conclusions

Molecular classes of $Ca^{2+}$ channel $\alpha_1$-subunits show extensive matches with functional types of $Ca^{2+}$ channels essentially based upon pharmacological sensitivities: three classes, $\alpha_{1S}$, $\alpha_{1C}$ and $\alpha_{1D}$, encode DHP-sensitive L-types and the other three classes, $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1E}$ (BI, BIII and BII), encode DHP-insensitive P/Q, N and R types, respectively. The P/Q-$\alpha_{1A}$, N-$\alpha_{1B}$ and R-$\alpha_{1E}$ channels can be further distinguished by specific blocking agents, $\omega$-Aga-IVA, $\omega$-CgTx-GVIA and $Ni^{2-}$, at low concentrations. This extensive correspondence between molecular identity and pharmacological distinction may not be surprising and is in fact rather quite natural, if we consider the similarity in their direction of approach to understand biological phenomena. Both approaches dictate biological effects in molecular structure. A slight difference between them is that pharmacological effects are mapped on chemical structures of drugs but molecular biology can define biological phenotypes dependent upon amino acid sequences of proteins that are receptors for drugs.

Thus, the disciplines of pharmacology and molecular biology made their first encounter to open a new field, "molecular pharmacology". The scientific approach, especially molecular cloning and structure-function studies, carried out in numerous laboratories have contributed to a contemporary picture of various pharmacological action sites. Will a second encounter occur? This is in fact being embodied in transgenesis, which can disrupt an ensemble of proteins and other constituents within the living body by transforming specific genetic information. Characterization of animal models themselves with transgene (or gene disruption) of drug receptors and their reaction to drugs at the level of the whole animal body or tissue, as done in classical pharmacology, has already started. There is no doubt that this research area together with structural biology will form a future cutting edge in pharmacology.

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