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Molecular Pharmacology of High Voltage-Activated Calcium Channels

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Voltage-gated calcium channels are key sources of calcium entry into the cytosol of many excitable tissues. A number of different types of calcium channels have been identified and shown to mediate specialized cellular functions. Because of their fundamental nature, they are important targets for therapeutic intervention in disorders such as hypertension, pain, stroke, and epilepsy. Calcium channel antagonists fall into one of the following three groups: small inorganic ions, large peptide blockers, and small organic molecules. Inorganic ions nonselectively inhibit calcium entry by physical pore occlusion and are of little therapeutic value. Calcium-channel-blocking peptides isolated from various predatory animals such as spiders and cone snails are often highly selective blockers of individual types of calcium channels, either by preventing calcium flux through the pore or by antagonizing channel activation. There are many structure-activity-relation classes of small organic molecules that interact with various sites on the calcium channel protein, with actions ranging from selective high affinity block to relatively nondiscriminatory action on multiple calcium channel isoforms. Detailed interactions with the calcium channel protein are well understood for the dihydropyridine and phenylalkylamine drug classes, whereas we are only beginning to understand the molecular actions of some of the more recently discovered calcium channel blockers. Here, we provide a comprehensive review of pharmacology of high voltage-activated calcium channels.

KEY WORDS: Calcium channels; conotoxins; agatoxins; dihydropyridines; phenylalkylamines; piperidines; SAR; farnesol; gabapentin; benzothiazepines.

INTRODUCTION

Calcium entry mediated by voltage-gated channels triggers a range of cellular responses such as the regulation of calcium-dependent second messenger cascades, calcium release from cytoplasmic stores, and gene expression (Dolmetsch *et al.*, 2001). Calcium ions contribute to neuronal excitability, neurite outgrowth, and neurotransmitter release within the nervous system (i.e., Dunlap *et al.*, 1995; Tsien *et al.*, 1988; Wheeler *et al.*, 1994), and trigger smooth muscle contraction. Excitable tissues express multiple types of voltage-gated calcium channels, each associated with specialized physiological roles.

Based both on their pharmacological and biophysical profiles, voltage-dependent calcium channels have been classified into T-, L-, N-, P-, Q-, and R-types (Bean, 1989; Hess, 1990; McCleskey, 1994; Nowycky *et al.*, 1985; Randall and Tsien, 1995; Tsien *et al.*, 1988, 1991; Zhang *et al.*, 1993). The low voltage-activated (LVA) T-type calcium channels typically activate at fairly negative potentials (−70 to −50 mV) (i.e., Akaike *et al.*, 1989; Bean, 1985; Lacinova *et al.*, 2000a; Takahashi *et al.*, 1991). By contrast, the family of high voltage-activated (HVA) calcium channels activates at more positive membrane potentials and includes L-, N- P-, Q-, and R-types (Catterall, 2000; Fox *et al.*, 1987). Although these calcium channels show overlapping biophysical properties,

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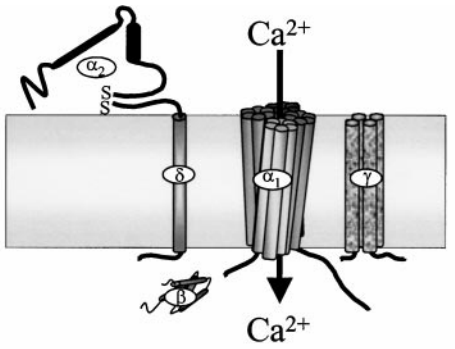
they can be distinguished by their unique pharmacology: L-type channels are highly sensitive to dihydropyridines (DHPs) (Bean, 1984; Droogmans and Callewaert, 1986; Striessnig, 1999); N-type channels are defined by their unique sensitivity to ω-conotoxin GVIA; P- and Q-type channels are identified via their specific but distinct sensitivities to ω-agatoxin IVA (for review, see Adams *et al.*, 1993); R-type calcium channels were originally defined by their resistance to these antagonists (Zhang *et al.*, 1993), but recently selective peptide blockers of these channels have been identified (Bourinet *et al.*, 2001; Newcomb *et al.*, 1998). Multiple isoforms of R-type calcium channels with distinct pharmacologies have now been described (Tottene *et al.*, 1996).

In the nervous system, different types of HVA calcium channels serve specialized cellular functions and have varying subcellular distributions. For example, L-type calcium channels are typically confined to cell bodies (Hell *et al.*, 1993) where they likely regulate calcium-dependent enzymes and gene expression. N- and P/Q-type calcium channels show highest expression levels at presynaptic terminals and are directly coupled to neurotransmitter release (Wheeler *et al.*, 1994; Westenbroek *et al.*, 1992; 1995). R-type calcium channels are expressed on proximal dendrites and may, in some neurons, participate in neurotransmitter release (Hanson and Smith, 2002). In view of their importance in neurophysiology, one might expect the loss of calcium channel activity to result in severe consequences for neuronal function. For example, loss of (or reduction in) P/Q-type calcium channel activity has been linked to etiologies such as absence seizures, cerebellar ataxia, and migraine (Fletcher *et al.*, 1996; Kraus *et al.*, 2000; Ophoff *et al.*, 1996; Wappl *et al.*, 2002). Malfunction or loss of retinal and cochlear L-type calcium channels results in congenital stationary night blindness and deafness, respectively (Bech-Hansen *et al.*, 1998; Platzer *et al.*, 2000). By contrast, mice lacking N-type calcium channels are normal but display hyposensitivity to pain (Kim *et al.*, 2001a; Saegusa *et al.*, 2001). Mice lacking certain T-type calcium channel isoforms also appear normal, but display reduced seizure activity (Kim *et al.*, 2001b). It is therefore not surprising that N- and T-type calcium channels have become important pharmacological targets for the development of analgesics and antiepileptics (Alps, 1992; Cox, 2000; Enyeart *et al.*, 1990; Peters *et al.*, 1991; Vanegas and Schaible, 2000;). Outside of the nervous system, blockers of smooth muscle L-type calcium channels have been targeted in the treatment of hypertension (Kwan and Wang, 1993; Weinberger, 1992). Thus, understanding the molecular pharmacology of these channels remains of fundamental importance.

MOLECULAR BIOLOGY OF CALCIUM CHANNELS

HVA calcium channels are comprised of as many four distinct subunits (α_1, α_2-δ, β, γ; for review, see Catterall, 2000). The α_1 subunit can form a functional calcium channel with regard to permeation, activation and inactivation characteristics, and pharmacology. It comprises four homologous domains (termed I through IV), each of which contains six transmembrane helices (S1–S6), as well as a p-loop motif (Catterall, 2000; Fig. 1). The ancillary α_2-δ, β, and γ subunits modulate functional properties of the α_1 subunit, such as activation and inactivation characteristics, and appear to participate in targeting the α_1 subunit to the cell membrane (Castellano *et al.*, 1993a,b; Felix, 1999; Klugbauer *et al.*, 1999; Lacerda *et al.*, 1991; Rousset *et al.*, 2001; Sharp *et al.*, 2001; Stea *et al.*, 1993; Zamponi *et al.*, 1996b). Ten genes encoding for nine neuronal calcium channel α_1 subunits (termed α_{1A} through α_{1I}) and the skeletal muscle L-type channel (α_{1S}), four genes encoding distinct β subunits (termed β_1 through β_4), four genes encoding the α_2-δ complex, and seven genes encoding different γ subunits have been cloned and functionally expressed (for review, see Birnbaumer *et al.*, 1994; Chu *et al.*, 2001; Ellis *et al.*, 1988; Kim *et al.*, 1992; Stea *et al.*, 1995; Williams *et al.*, 1992b). On the basis of their pharmacological and biophysical profiles, cloned calcium channel α_1 subunits have been correlated with their native counterparts (Fig. 1): α_{1A} likely encodes both P- and Q-type channels (Bourinet *et al.*, 1999; Mori *et al.*, 1991; Sather *et al.*, 1993; Stea *et al.*, 1994a,b); α_{1B} encodes an N-type calcium channel (Dubel *et al.*, 1992; Fujita *et al.*, 1993; Stea *et al.*, 1993; Williams *et al.*, 1992a); α_{1C}, α_{1D}, and α_{1F} are L-type channels (Bech-Hansen *et al.*, 1998; Mikami *et al.*, 1989; Snutch *et al.*, 1991; Tomlinson *et al.*, 1993; Williams *et al.*, 1992a); α_{1E} likely constitutes a variant of the R-type calcium channel family (Matsuda *et al.*, 2001; Soong *et al.*, 1993; Tottene *et al.*, 1996; Williams *et al.*, 1994; Wilson *et al.*, 2000); and, α_{1G}, α_{1H}, and α_{1I} are T-type calcium channels (Beedle *et al.*, 2002; Cribbs *et al.*, 1998, 2000; Lee *et al.*, 1999; McRory *et al.*, 2001; Monteil *et al.*, 2000; Perez-Reyes *et al.*, 1998). More recently, a new nomenclature for calcium channels has been introduced (Ertel *et al.*, 2000, see Fig. 1).

Although the ancillary calcium channel subunits can modulate the pharmacological properties of the channels (Gee *et al.*, 1996; Nishimura *et al.*, 1993; Williams *et al.*, 1992b; Zamponi *et al.*, 1996a,b), the overall pharmacological profile of a given calcium channel subtype is primarily defined by the α_1 subunit. It contains binding sites for multivalent cations (Cloues *et al.*, 2000; Zamponi *et al.*, 1996a), interaction sites for peptide blockers (i.e.,



α_1	170-250 kDa
$\alpha_2\delta$	~170 kDa
β	50-78 kDa
γ	~36 kDa

Super Family	Family	Former Name	Proposed Name	Defining Pharmacologies
HVA	L	α_{1C}	$Ca_v1.2$	DHPs
		α_{1D}	$Ca_v1.3$	
		α_{1F}	$Ca_v1.4$	
		α_{1S}	$Ca_v1.1$	
	N	α_{1B}	$Ca_v2.2$	ω-conotoxin GVIA
	P/Q	α_{1A}	$Ca_v2.1$	ω-agatoxin IVA
	R	α_{1E}	$Ca_v2.3$	SNX482
LVA	T	α_{1G}	$Ca_v3.1$	no selective blockers reported to date
		α_{1H}	$Ca_v3.2$	
		α_{1I}	$Ca_v3.3$	

Fig. 1. Generalized structure, nomenclature, and pharmacology of voltage-gated calcium channels. The pore-forming α_1 subunit consists of four domains, each of which comprises of six transmembrane helices, and is responsible for the activation and inactivation properties of the channel. These properties are further modulated by ancillary α_2-δ, β, and γ subunits, which also help to target the α_1 subunit to the cell membrane. The nomenclature for various α_1 subunits, along with defining pharmacologies, are shown.

Bourinet *et al.*, 2001; Ellinor *et al.*, 1994; Feng *et al.*, 2001) and, perhaps most important from a therapeutic point of view, a number of receptor sites for small organic molecules (see Glossmann and Striessnig, 1990; Striessnig *et al.*, 1994; Zamponi, 1997). In the ensuing sections, we shall discuss molecular aspects of interactions between calcium channel blockers and HVA calcium channels.

INORGANIC DIVALENT AND TRIVALENT CATIONS

The ability of metal ions to block voltage-gated calcium channels is well established. Cadmium ions are useful tools to nonspecifically block all types of HVA calcium channels (i.e., Akaike *et al.*, 1978; Byerly *et al.*, 1985; Hagiwara and Byerly, 1981; Hagiwara and Takahashi, 1967; Kostyuk *et al.*, 1977; Lansman *et al.*, 1986). By contrast, nickel ions were once considered specific blockers of T-type calcium channels (Fox *et al.*, 1987; Lee *et al.*, 1999; Mlinar and Enyeart, 1993; for review, see Hille, 1992) and were widely used to discriminate T-type currents from other calcium currents. However, a number of HVA calcium channel subtypes are potently inhibited by nickel. For example, both transiently expressed (Zamponi *et al.*, 1996a) and native R-type calcium channels can be potently inhibited by nickel (Tottene *et al.*, 1996), and at high enough concentrations (i.e., in the hundred micromolar range), nickel blocks transiently expressed N-type, L-type and P/Q-type calcium channels (Zamponi

et al., 1996a). After the cloning and expression of three different genes encoding T-type calcium channels, it became clear that high affinity nickel block is not necessarily seen with all T-type calcium channels. While tight nickel block is observed with α_{1H} channels, α_{1G} and α_{1I} channels are blocked with much lower affinity (Lee *et al.*, 1999). As a consequence, the use of nickel ions to discriminate between T-type and non-T-type calcium channels is no longer warranted.

Several other metal ions such as zinc, lead, copper, and aluminum have also been reported to block calcium currents (i.e., Busselberg *et al.*, 1991, 1992; Lansman *et al.*, 1986). Moreover, many types of trivalent ions such as lanthanum, yttrium, and holmium are potent inhibitors of calcium channels (Beedle *et al.*, 2002; Biagi and Enyeart, 1990; Block *et al.*, 1998; Lansman, 1990; Mlinar and Enyeart, 1993) with blocking affinities in the low nanomolar range. Under identical experimental conditions (i.e., transiently expressed calcium channels in 2 mM barium), yttrium, one of the most potent trivalent cations, does not appear to discriminate between HVA and LVA calcium channels (Beedle *et al.*, 2002), suggesting that the site of action of these ions is conserved across all types of voltage-gated calcium channels.

The primary mode of action of multivalent metal ions appears to be a physical occlusion of the permeation pathway (i.e., Lansman *et al.*, 1986). Data obtained with cadmium ions show a ring of glutamic acid residues in the p-loop region is critical for pore block (Cloues *et al.*, 2000), and it is likely that this mechanism applies to all types of multivalent metal ions. However, for several multivalent metal ions, a second mode of action has been reported. A number of divalent cations exert effects on calcium channel gating by shifting the threshold for calcium channel activation towards more depolarized potentials (Busselberg *et al.*, 1994; Byerly *et al.*, 1985; Pekel *et al.*, 1993). This phenomenon was examined in detail for nickel block of transiently expressed R-type calcium channels (Zamponi *et al.*, 1996a). The authors showed this alternative blocking action was likely mediated by nickel binding to a site distinct from the pore blocking site, whose occupancy antagonizes channel opening. Interestingly, this effect is observed with all HVA calcium channel isoforms from rat brain (Zamponi *et al.*, 1996a), and with transiently expressed α_{1H} T-type calcium channels (Lee *et al.*, 1999), but the magnitude and nickel concentrations required to mediate this effect vary with channel type. More recently, profound acceleration of inactivation rates by yttrium on transiently expressed P/Q-type (and to a lesser extent L-type) calcium channels have been reported (Beedle *et al.*, 2002). These effects are not correlated with the pore blocking affinity of the ions, indicating that they occur through actions on a separate site allosterically linked to the inactivation machinery, but the exact identities of these modulatory sites remain enigmatic.

In summary, many multivalent ions block voltage-gated calcium channels, but they may do so in a nonselective manner. For this reason, as well as their toxicities, such ions do not serve as clinically useful calcium channel antagonists.

PEPTIDE BLOCKERS

Many types of voltage-gated and ligand-gated ion channels are inhibited by peptide blockers that occur naturally in venoms of predatory animals such as snakes, scorpions, spiders, and cone snails. In this regard, calcium channels are no exception. Indeed, some of the most specific inhibitors of voltage-gated calcium channels are peptide toxins isolated from fish-hunting marine snails (i.e., conotoxins) and funnel web spiders (i.e., agatoxins). The blockers have been used extensively as molecular tools for the identification and isolation of individual calcium channel subtypes, and more recently, they have emerged as potential therapeutics in the treatment of disorders such as pain (i.e., Heading, 1999).

Conotoxins

Numerous calcium-channel-blocking peptides (20–30 amino acids) have been isolated from the venoms of several different species of cone snails (Olivera *et al.*, 1985). Their structures are rigid, as they are spatially constrained by disulfide bonds, thereby providing the potential for a high degree of specificity for target interactions. Besides blocking calcium channels (i.e., ω-conotoxins), the family of conotoxins include, among others, blockers of skeletal muscle sodium channels (μ-conotoxins; i.e., Becker *et al.*, 1992; French *et al.*, 1996) and blockers of the acetylcholine receptor (α-conotoxins, i.e., Kulak *et al.*, 1997).

The archetypal calcium-channel-blocking conopeptide, ω-conotoxin GVIA, is a 27 amino acid peptide isolated from the fish-hunting *Conus geographus* sea snail (Olivera *et al.*, 1994). This peptide is highly selective for N-type calcium channels from a variety of species and nerve tissues (but see Williams *et al.*, 1992a), causing virtually irreversible block in the mid nanomolar range (i.e., Boland *et al.*, 1994; Ellinor *et al.*, 1994; Feng *et al.*, 2001; Stocker *et al.*, 1997). *Conus geographus* produces several additional ω-conotoxins (i.e., GVIB, GVIC, GVIIA, and GVIIB, see Hillyard *et al.*, 1992; Olivera *et al.*, 1994),

however their actions on N-type calcium channels remains poorly characterized.

The *Conus magus* snail also produces several calcium channel blocking peptides, including ω-conotoxins MVIIC and MVIIA (Olivera *et al*., 1994). Similar to observations with GVIA, MVIIA (25 amino acids) selectively and almost irreversibly blocks N-type calcium channels (i.e., Feng *et al.*, 2001; Fox, 1995; Woppmann *et al.*, 1994). A synthetic version of MVIIA (termed SNX-111 or more recently, Ziconotide, Elan Pharmaceuticals) is currently in phase three clinical trials for the treatment of severe pain (Ridgeway *et al.*, 2000; Wang *et al.*, 2000). However, the requirement for intrathecal injection limits its usefulness and the potential for severe side effects has tempered the initial enthusiasm for Ziconotide as a potent analgesic (Penn and Paice, 2000). The occurrence of these side effects may be due to incomplete selectivity of MVIIA for N-type channels (Feng *et al.*, 2003; see also Lewis *et al.*, 2000) and has triggered the search for more selective N-type calcium channel blocking conotoxins. Recently, ω-conotoxin CVID, a structural analogue to MVIIA, has been isolated from *Conus catus* (Lewis *et al.*, 2000). This peptide is highly selective for N-type channels and is currently in phase two clinical trials in Australia. Unlike ω-conotoxin MVIIA and its derivatives, MVIIC (26 amino acids) and related toxin SVIB (26 amino acids, isolated from *Conus striatus*) reversibly block N-type, P-type, and Q-type calcium channels (Bourinet *et al.*, 1999; Grantham *et al.*, 1994a; Hillyard *et al.*, 1992; McDonough *et al.*, 1997a; Nielsen *et al.*, 1996; Sather *et al.*, 1993; Woppmann *et al.*, 1994), whereas L-type and T-type channels are insensitive.

Agatoxins

A group of peptide neurotoxins has been isolated from the venom of the American funnel web spider, *Agenelopsis aperta*, which includes blockers of glutamate receptors (α-agatoxins) and activators of sodium channels (μ-agatoxins). Among calcium-channel-inhibiting agatoxins (i.e., ω-agatoxins; Olivera *et al.*, 1994), four subclasses have been identified, three of which (ω-agatoxin II, III, and IV) have been shown to inhibit neuronal calcium channels (Adams *et al.*, 1993; Olivera *et al.*, 1994). Like the conotoxins, the ω-agatoxins are spatially constrained by several disulfide bonds, but tend to be substantially larger (40–80 amino acids) and display more variability in size and in structure.

ω-agatoxin IIIA (78 amino acids) has been shown to inhibit a range of different calcium channel subtypes, including cardiac and neuronal L-type channels, N-type channels, and both P- and Q-type calcium channels, but it does not appear to block T-type channels (Mintz, 1994; Mintz *et al.*, 1991). In contrast, ω-agatoxin IVA (48 amino acids) is considered a specific blocker of P- and Q-type calcium channels (Mintz *et al.*, 1992). However, low affinity block of N-type calcium channels has been reported (Sidach and Mintz, 2000) and there may be a small degree of block of human α_{1E} channels (Williams *et al.*, 1994). While P-type channels are blocked at low nanomolar concentrations, Q-type channels require about 100-fold higher concentrations for block (i.e., Bourinet *et al.*, 1999). To date, ω-agatoxin IVA remains the only selective blocker of P/Q-type calcium channels.

Novel Toxins

A number of novel toxins with calcium-channel-blocking affinity have been identified. DW13.3., a toxin isolated from the venom of the spider *Filistata hibernalis*, resembles ω-agatoxin IIIA in its blocking action, inhibiting native and transiently expressed L-, P-, and N-type channels (Sutton *et al.*, 1998). SNX-325, a novel peptide isolated from the venom of the spider *Segestria florentina*, has been reported to selectively block-cloned N-type α_{1B}, but not L-type cardiac α_{1C}, P/Q-type α_{1A} or R-type α_{1E} channels (Newcomb *et al.*, 1995).

Two additional toxins have been isolated from tarantula venom, ω-grammotoxin SIA from *Grammostola spatulata* (Lampe *et al.*, 1993; Piser *et al.*, 1994, 1995; Turner *et al.*, 1995) and SNX-482 from *Hysterocrates gigas* (Newcomb *et al.*, 1998). At low micromolar concentrations, ω-grammotoxin reversibly blocks N-, P-, and Q-type calcium channels, but has no effect on L-type calcium channels (Piser *et al.*, 1995; McDonough *et al.*, 1997a). Furthermore, this toxin inhibits an as yet uncharacterized "resistant" calcium current in rat brain synaptosomes (Turner *et al.*, 1995). SNX-482 was originally considered a selective blocker of R-type calcium channels (Newcomb *et al.*, 1998), but more recent evidence suggests that L-type calcium channels are also inhibited (Bourinet *et al.*, 2001), although block of the L-type channels was shown to be incomplete and at lower affinity compared with R-type channels.

Besides spiders and marine snails, calcium-channel-blocking peptides have also been isolated from snakes and scorpions. Calcicludine, found in green mamba toxin, structurally resembles potassium-channel-blocking dendrotoxins (Gilquin *et al.*, 1999), and blocks L-type calcium channels (Schweitz *et al.*, 1994). This toxin also shifts the half-activation potential of non-L-type channels toward more hyperpolarized potentials, thus upregulating

current activity (Stotz *et al.*, 2000). Kurtoxin, isolated from scorpions, was originally reported to selectively inhibit T-type calcium channels (Chuang *et al.*, 1998), however, more recent evidence suggests that N- and L-type calcium channels are also partially inhibited (Sidach and Mintz, 2002). Hence, a truly selective T-type calcium channel peptide blocker remains elusive. Glycerotoxin, Isolated from Glycera convoluta, stimulates neurosecretion by promoting activation of N-type channels. (Meunier *et al.*, 2002).

Molecular Basis of Toxin Block

With regard to their blocking actions, peptide blockers of voltage-gated calcium channels appear to come in two flavors—gating modifiers and pore blockers. Gating modifier toxins prevent calcium channel activation likely by antagonizing voltage-sensor movement. This class of toxins includes ω-agatoxin IVA (McDonough *et al.*, 1997b), ω-grammotoxin SIA (McDonough *et al.*, 1997a), SNX-482 (Bourinet *et al.*, 2001), and kurtoxin (Sidach and Mintz, 2002). Block by these toxins is typically poorly reversible following washing, but channel activity can almost completely be recovered following strong repetitive membrane depolarizations, suggesting that voltage-sensor movement might physically dislodge the toxin from the docking site (Bourinet *et al.*, 2001; McDonough *et al.*, 1997a; Mintz *et al.*, 1992). Although detailed information about the underlying channel structural requirements is still lacking, there is evidence that ω-agatoxin IVA interacts, at least in part, with the α_{1A} domain IV S3-S4 region (Bourinet *et al.*, 1999). Insertion of an asparagine and proline into this region through alternative splicing shifts the ω-agatoxin sensitivity from high affinity (i.e., P-type calcium channel-like) to lower affinity seen with native Q-type channels (Bourinet *et al.*, 1999). Moreover, a single glutamic acid residue in this region seems to be essential for ω-agatoxin IVA block (Winterfield and Swartz, 2000). Consistent with an involvement of domain IV in the action of gating modifiers, block of R-type calcium channels by SNX-482 is critically dependent on the presence of α_{1E} domains III and IV. Substitution of both of these domains into the L-type calcium channel α_1 subunit confers all aspects of SNX-482 block (Bourinet *et al.*, 2001). In general, the S3–S4 linker region appears to be an important determinant of gating modifier toxin action, as similar structural determinants appear to be important to gating block of potassium channels (Li-Smerin and Swartz, 1998, 2001).

By contrast, ω-conotoxins GVIA, MVIIA, MVIIC, and SVIB, as well as ω-agatoxin IIIA appear to act by physically occluding the channel pore. Consistent with a distinct mechanism of action from the gating modifiers, ω-grammotoxin SIV cannot be displaced by the pore blocker ω-conotoxin GVIA (Lampe *et al.*, 1993). A partial pore block is seen with ω-agatoxin IIIA. Even at saturating concentrations, ω-agatoxin IIIA produces incomplete block of P-type and N-type calcium channels (Mintz, 1994). The remaining current through N-type channels cannot be abolished upon subsequent application of the pore blocker ω-conotoxin GVIA, suggesting that ω-agatoxin IIIA may act by incompletely occluding the pore (McDonough *et al.*, 1995). In contrast, ω-agatoxin IVA completely abolishes the P-type current remaining in the presence of ω-agatoxin IIIA, consistent with the notion that the two toxins act by distinct mechanisms. The site of action for GVIA block appears to be localized predominantly to the domain III region. Site-directed mutagenesis has identified crucial components of the binding site of ω-conotoxin GIVA within the external vestibule of the α_{1B} channel, in particular, a number of amino acid residues in the S5–S6 region of domain III are implicated (Ellinor *et al.*, 1994). Additional mutagenesis in this region (Feng *et al.*, 2001) showed that replacement of glycine 1326 with proline resulted in a dramatic acceleration in the blocking rate constant for GVIA and MVIIA action. Block became fully reversible, suggesting that residue 1326 may form a barrier that restricts access of the toxin to the blocking site, but also stabilizes the bound toxin. Consistent with the observation that both GVIA and MVIIA were similarly affected, ω-conotoxin MVIIA (and MVIIC for that matter) have been shown to compete with ω-conotoxin GVIA in biochemical assays (McDonough *et al.*, 1995; Olivera *et al.*, 1994).

Overall, it appears that certain calcium channel α_1 subunits contain two distinct binding sites for peptide toxins: first, a macrosite near the outer mouth of the pore which seems to interact with the majority of these toxins; and, secondly, a distinct site which interacts with gating modifying toxins. Although some progress has been made in identifying the channel structural determinants of toxin block of voltage-gated calcium channels, as summarized in Fig. 2, much work remains to precisely identify the interactions between individual toxin side chains and amino acid residues on the channel molecule.

SMALL ORGANIC MOLECULES

Numerous small organic molecules having agonistic/antagonistic effects on calcium channels have been identified and they fall into various structure-activity-relation (SAR) classes, summarized in Fig. 3. For example, L-type calcium channel α_1 subunits contain at least seven distinct receptor sites for various SAR drug

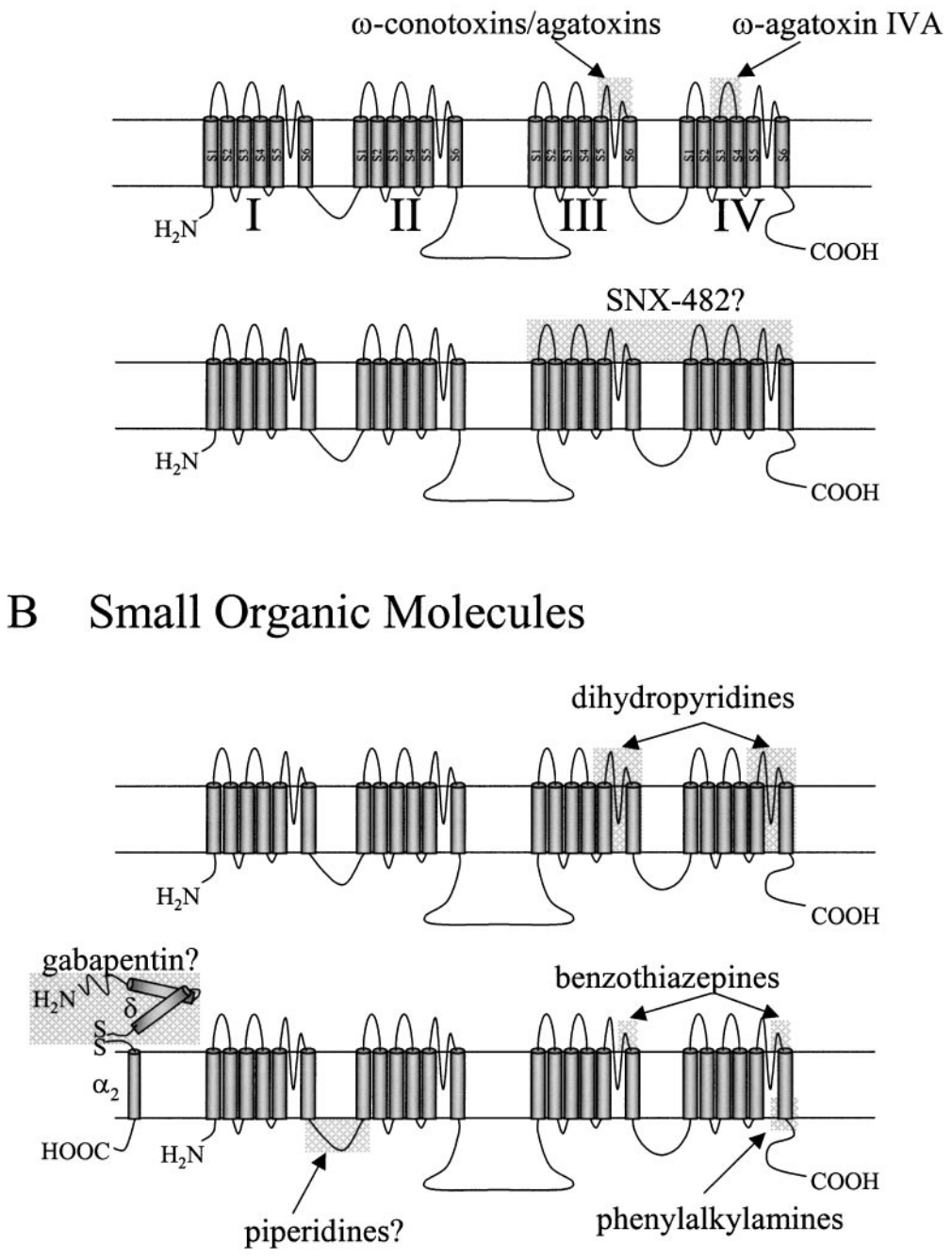


Fig. 2. Possible interactions of pharmacologically active voltage-gated calcium channel antagonists with the channel. The interaction sites are not necessarily all-inclusive, as other sites may be uncharacterized at this time. A) Pore-blocking peptides ω-conotoxins GVIA, MVIIA, MVIIC, and SVIB, and ω-agatoxin IIIA have been shown to interact with residues in the extracellular portion of domain III S5–S6 linker, while the gating modifier ω-agatoxin IVA has been shown to interact with the α_{1A} IV S3–S4 linker. Domains III and IV appear to be necessary for SNX-482 interaction with the channel, although precise details remain unknown. B) Various SAR classes of small organic molecules interact with voltage-gated calcium channels. Dihydropyridines appear to interact with S5–S6 regions in domains III and IV, while the extracellular portions of S5–S6 linkers in domains III and IV are important for interactions with benzothiazepines. Phenylalkylamines are thought to target the IV S6 region intracellularly. Evidence suggests the site of piperidine action might involve the intracellular I-II linker. Gabapentin possibly interacts with the α_2 ancillary subunit, rather than directly with the α_1 subunit.

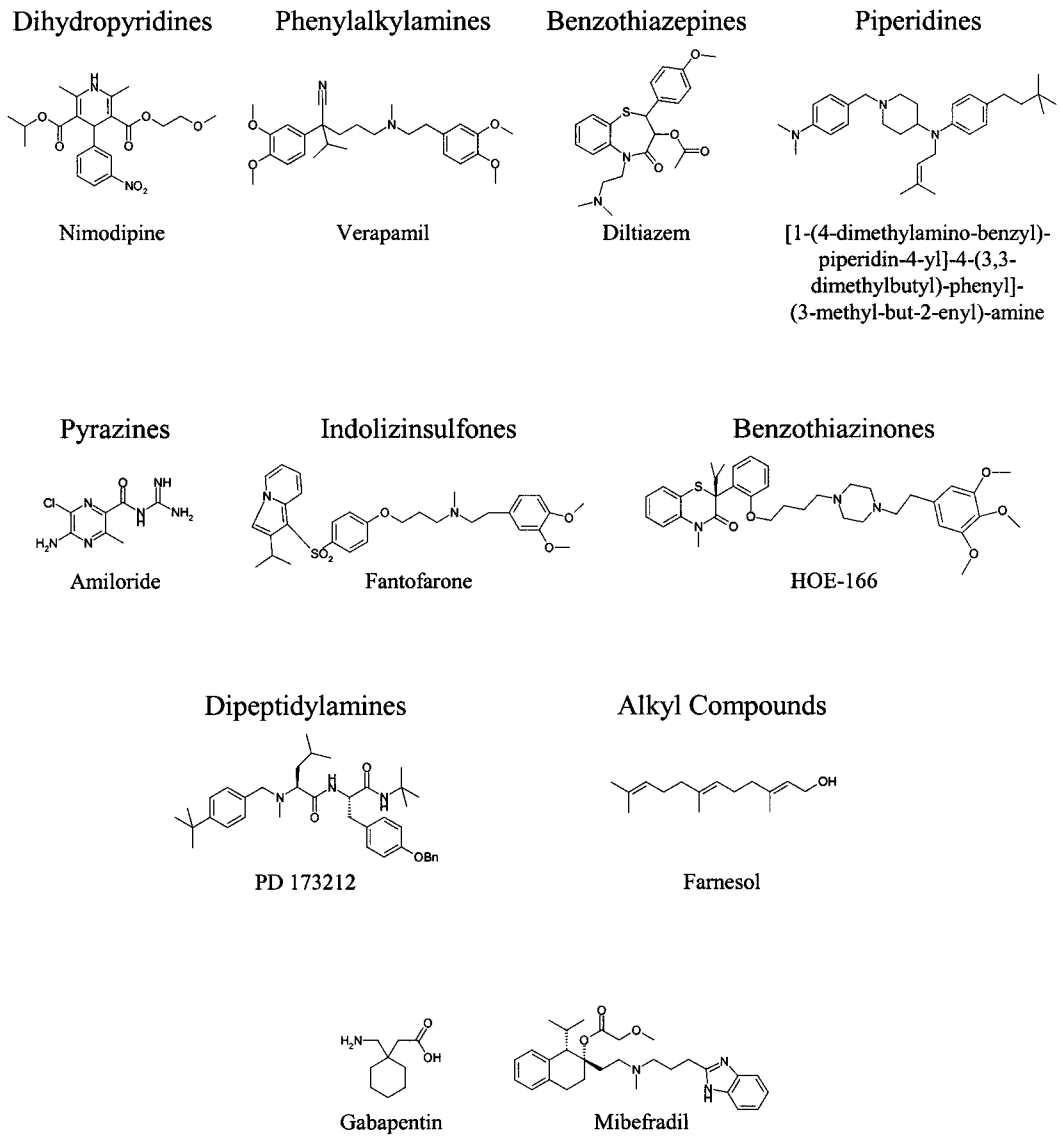


Fig. 3. Examples of calcium channel blockers from different SAR classes.

classes (Striessnig *et al.*, 1994; Zamponi, 1997), including the well-characterized sites for dihydropyridines (DHPs), phenylalkylamines, and benzothiazepines (see Fig. 2). In addition, the existence of unique receptor sites for pyrazines, piperidines, indolizinsulfones, and benzothiazinones has been suggested (Glossmann and Striessnig, 1990; Striessnig *et al.*, 1994). More recently, a number of calcium-channel-blocking molecules with unique structural features have been identified, suggesting the presence of additional, yet to be identified, drug-binding sites on the calcium channel protein (for review, see Snutch *et al.*, 2001). Of all small organic calcium channel inhibitors, DHPs are the most widely used clinically, and consequently, they are the best characterized class of calcium channel antagonists, from both a drug and channel structural point of view. However, with N-type calcium channels emerging as a key target in pain treatment (Cox, 2000), substantial efforts in understanding the action of small organic molecules on these channels are underway.

Dihydropyridines

Dihydropyridines constitute a group of small organic compounds which are based on a core pyridine structure, and include compounds such as nifedipine, nimodipine,

nitrendipine, isradipine, and BAY K 8644 (i.e., Bangalore *et al.*, 1994; Sun and Triggle, 1995). Their ability to block or enhance calcium currents has been well documented (i.e., Bangalore *et al.*, 1994; Bean, 1984; Bechem and Hoffmann, 1993; Droogmans and Callewaert, 1986; Peterson and Catterall, 1995). Although they are considered to be specific blockers of L-type calcium channels, T-type channels may also show sensitivity to certain commonly used DHPs (Akaike *et al.*, 1989; Romanin *et al.*, 1992; Santi *et al.*, 1996). Recently, members of a novel class of nifedipine analogues have also been shown to preferentially block α_{1G} T-type channels (Kumar *et al.*, 2002). DHPs can discriminate among different types L-type calcium channels, with α_{1C} channels typically showing higher affinity block compared with α_{1D} (Eller *et al.*, 2000; Koschak *et al.*, 2001; Xu and Lipscombe, 2001).

A key feature associated with DHP block is a strong state dependence. Whereas L-type channels are often poorly DHP sensitive at a hyperpolarized membrane potential, blocking affinity can increase, by several orders of magnitude, at more depolarized membrane potentials, as evidenced by a dramatic leftward shift in the position of the steady state inactivation curve following DHP application (i.e., Kumar *et al.*, 2002). Moreover, some DHPs, such as isradipine, promote a speeding of the time course of current decay which has led to the suggestion that DHPs might promote a drug-induced inactivated conformation (Eller *et al.*, 2000), but it is likely that open channel block also contributes to this effect (Handrock *et al.*, 1999; Lacinova *et al.*, 2000b). It has also been shown that the inactivated conformation of the channel favours certain subclasses of DHPs, suggesting that specific functional groups on the DHP molecule control state dependence of block (Sun and Triggle, 1995). However, the detailed molecular basis for DHP action on L-type channel inactivation remains only poorly understood.

The location of the DHP binding site on the skeletal muscle L-type channel α_1 subunit was originally identified by tryptic cleavage of the channel protein and photoaffinity labelling (Striessnig *et al.*, 1991). It was revealed that the receptor site comprises of two separate regions, i.e., the S5–S6 regions of domains III and IV, as illustrated in Fig. 2. Moreover, permanently charged membrane impermeant DHP derivatives were found to inhibit calcium channel activity, further supporting the notion of a binding site located close to the external side of the channel (Bangalore *et al.*, 1994; Kass *et al.*, 1991; Kwan *et al.*, 1995; Strubing *et al.*, 1993). By creating chimeric calcium channels between α_{1S} and α_{1A}, DHP sensitivity was conferred onto α_{1A} (Grabner *et al.*, 1996), but only when the S5, S6, and pore lining S5–S6 regions of domains III and IV all were substituted into α_{1A}. To confer the stimulatory action of BAY K 8644 onto α_{1A}, the additional insertion of the domain IV S6 region was required. Binding sites for DHP compounds were subsequently further defined using site-directed mutagenesis. Mutation of a threonine residue in the III S5 region of α_{1C} abolished both agonistic (Bay K 8644) and antagonistic (isradipine) DHP effects, while mutation of a proximal glutamine residue reduced the isradipine affinity without affecting BAY K 8644 sensitivity (Ito *et al.*, 1997). Additionally, several amino acid residues in the domain III S5, III S6, and IV S6 regions have been shown to affect DHP block of L-type calcium channels (Wappl *et al.*, 2001; Yamaguchi *et al.*, 2000) and are able to confer full DHP sensitivity to α_{1A} calcium channels (Sinnegger *et al.*, 1997). Hence, the overall DHP site appears to comprise several microsites located in domain III and IV, and different antagonists and agonists appear to require interactions with different amino acid residues within these microsites. On the basis of the identified residues, and on the crystal structure of the KcsA potassium channel, a three-dimensional structural model of the DHP binding site on the L-type calcium channel α_1 subunit has been devised (Huber *et al.*, 2000), but the authors did not attempt to determine the orientation of DHPs within this binding pocket. Hence, how residues comprising the site interact with specific R groups on the DHP molecule is unknown. Furthermore, because the residues comprising the L-type channel DHP receptor site are not conserved in T-type channels, it is not known what the channel or drug structural determinants of DHP action are for T-type calcium channels. Hence, while DHP action on voltage-gated calcium channels has been subject to intense investigation for almost two decades, significant gaps in our understanding of their molecular interactions with calcium channels remain.

Phenylalkylamines

Phenylalkylamines are structurally related to local anaesthetics, in the sense that they consist of at least one phenyl ring linked to an amine head group by an alkyl chain, and include compounds such as verapamil, devapamil, D888, and D600. Although they are widely thought to be preferential L-type channel inhibitors, they also block N-type and P/Q-type calcium channels (Diochot *et al.*, 1995; Hering *et al.*, 1989; Lee and Tsien, 1983; McDonald *et al.*, 1984). For example, transiently expressed L-type calcium channels exhibit only about a 10-fold higher sensitivity to D600 than P/Q-type channels α_{1A} channels (Döring *et al.*, 1996), and N-type calcium channels also appear to show some sensitivity to

phenylalkylamines (Hockerman *et al.*, 1995). However, as with DHPs, block of L-type channels by phenylalkylamines is strongly dependent on membrane potential (Hering *et al.*, 1997; Sokolov *et al.*, 1999, 2001), thus impacting on the degree of drug selectivity among different calcium channels.

The approximate location of the phenylalkylamine receptor site was originally mapped to the S6 region of domain IV via photoaffinity labeling (Striessnig *et al.*, 1990). Transfer of the domain IV S6 region of α_{1S} into α_{1A} confers phenylalkylamine sensitivity (Döring *et al.*, 1996). Subsequent analysis using chimeric and mutant calcium channel α_1 subunits resulted in the identification of individual amino acid residues that participate in the formation of the phenylalkylamine receptor site. Mutagenesis of three amino acid residues in the α_{1C} IV S6 region (Y1463, A1467, I1470) attenuates phenylalkylamine sensitivity (Hockerman *et al.*, 1995). Since these residues are located near the cytoplasmic side of the channel, this suggests that phenylalkylamines probably diffuse across the cell membrane to reach the blocking site. Interestingly, the location of the phenylalkylamine binding site is reminiscent of that of the local-anaesthetic site in sodium channels (Ragsdale *et al.*, 1994), suggesting that block of calcium and sodium channels by local anaesthetic-like compounds may occur via similar mechanisms.

Benzothiazepines

The benzothiazepine receptor site interacts with structurally unique clinical agents such as diltiazem and tetrandrine, both of which are used to treat cardiac conditions such as angina (Hering *et al.*, 1993; King *et al.*, 1988; Prinz and Striessnig, 1993; Seydl *et al.*, 1993; for review, see Kaczorowski *et al.*, 1994). Originally, benzothiazepines were considered to be selective blockers of L-type calcium channels, however, this is no longer supported by the literature. For example, tetrandrine also effectively blocks other neuronal calcium channel subtypes, including T-type calcium channels (Weinsberg *et al.*, 1994), and diltiazem blocks transiently expressed L-type, R-type and P/Q-type channels with similar affinities (Cai *et al.*, 1997). Interestingly, however, only L-type calcium channels exhibit state-dependent inhibition, indicating that the detailed mechanisms of L-type channel block are distinct from those seen with non-L-type channels. Membrane impermeant benzothiazepine analogs retain their ability to block calcium channels (Hering *et al.*, 1993; Seydl *et al.*, 1993), suggesting the benzothiazepine receptor site is accessible directly from the external side of the channel (Kurokawa *et al.*, 1997). Data obtained via photoaffinity labeling of tryptic fragments of L-type calcium channels suggest that the benzothiazepine-binding site could, at least in part, be made up by portions of the domain III S5–S6 linker and by parts of the domain IV S6 region (Watanabe *et al.*, 1993; Kraus *et al.*, 1996), as illustrated in Fig. 2. Subsequent mutagenesis studies revealed that transfer of as few as three residues from L-type to P/Q-type calcium channels is sufficient to induce susceptibility to diltiazem action (Hering *et al.*, 1996; Kraus *et al.*, 1998). Moreover, transfer of domains III and IV of α_{1C} into α_{1A} confers the state-dependent properties of L-type channel block (Cai *et al.*, 1997). Hence, there appears to be some overlap in the channel structural determinants of DHP and benzothiazepine block, but the exact residues that are involved differ, consistent with biochemical and electrophysiological evidence suggesting that the receptor sites for these two SAR classes are distinct, but in close proximity to one other (Brauns *et al.*, 1995).

Piperidines

Diphenylbutylpiperidines were originally thought of as highly potent antagonists of the D_2 receptor and clinically used to treat various forms of psychosis (Seeman *et al.*, 1976; Seeman and Lee, 1975). However, there is now increasing evidence that this SAR class produces useful calcium channel antagonists with blocking affinities ranging from several nanomolar to micromolar ranges (Enyeart *et al.*, 1992; Galizzi *et al.*, 1986; Gould *et al.*, 1983; Grantham *et al.*, 1994b; King *et al.*, 1989; Sah and Bean, 1994; Santi *et al.*, 2002), with T-type calcium channels being the most potently inhibited channel isoform. More recently, potent block of N-type calcium channels by a piperidine derivative with oral analgesic activity has been reported (Hu *et al.*, 1999), suggesting that this drug class can perhaps be used to generate high affinity N-type calcium channel blockers.

Radioligand binding assays suggest that piperidines interact with a site on the calcium channel distinct from that for phenylalkylamines, benzothiazepines, and DHPs (for review, see Kaczorowski *et al.*, 1994), but the molecular identification of the drug receptor site has yet to be accomplished. There is evidence that point mutations in the calcium channel domain I-II linker region can alter the sensitivity of the channel to piperidines/piperazines (Zamponi *et al.*, 1996b). Moreover, the presence of G protein $\beta\gamma$ subunits known to bind to the I-II linker region of N-type calcium channel α_1 subunits result in a threefold increase in haloperidol affinity for N-type channels (Zamponi, 1999). However, it remains unclear whether the piperidine/piperazine receptor site comprises the I-II linker region or if the observed effects were mediated allosterically.

Other Calcium Channel Blocking SAR Classes

Blocking effects of the pyrazine diuretic amiloride on voltage-dependent calcium channels have been well documented. Amiloride is widely believed to preferentially interact with T-type calcium channels and has been used as a defining criterion of these channels (i.e., Avery and Johnston, 1996; Dzhura *et al.*, 1996; Mouginot *et al.*, 1997; Santi *et al.*, 1996; Wang *et al.*, 1996). However, its relatively low affinity (low millimolar range) and interactions with other cation channels and L-type calcium channels (i.e., Garcia *et al.*, 1990; Suarez-Kurtz and Kaczorowski, 1988) limit amiloride's usefulness. Although the exact site of action of amiloride remains unknown, it has been suggested to act as an external pore blocker (Kaczorowski *et al.*, 1994).

Indolizinsulfones and benzothiazinones each interact with unique receptor sites on the calcium channel α_1 subunit (Glossmann and Striessnig, 1990; Kenny *et al.*, 1993). The latter group revolves around the compound HOE 166, which has been shown to bind to L-type calcium channels (i.e., Glossmann and Striessnig, 1990; Striessnig *et al.*, 1994), but it is otherwise poorly described. Several studies have examined the interactions between voltage-gated calcium channels and indolizinsulfones. Fantofarone and derivative SR 33805 (Bois *et al.*, 1991; Gubin *et al.*, 1993; Kenny *et al.*, 1993; Schmid *et al.*, 1989) potently interact with cardiac and neuronal L-type calcium channels (Romey and Lazdunski, 1994; Romey *et al.*, 1994), but little remains known about their detailed action at the molecular level.

Dipeptidylamines are a novel class of calcium channel blockers that were identified through high-throughput screening of compound libraries with the goal of identifying novel N-type calcium channel blockers. N-type calcium channels in IMR32 cells are potently blocked by a compound formed through coupling of an N, N-di-substituted leucine and a tyrosine amine (termed PD173212) (Hu *et al.*, 1999). Among the derivatives of PD173212, blocking affinities as low as 40 nM were reported (Ryder *et al.*, 1999), making these compounds some of the highest affinity small organic N-type channel blockers identified to date. However, little information is available as to the affinities of these compounds on other types of calcium channels (and other types of ion channels, for that matter), and thus their therapeutic usefulness remains unknown.

N-type calcium channels are also potently inhibited by long alkyl chain molecules. The naturally occurring compound farnesol (an intermediate of the mammalian mevalonate pathway) selectively blocks N-type calcium channels among the HVA class with nanomolar affinity (Roullet *et al.*, 1999), with block appearing to occur via two mechanisms. At low concentrations, farnesol shifts the steady state inactivation curve toward more hyperpolarized potentials, indicative of strong inactivated channel block. At micromolar concentrations, farnesol mediates rapid open channel block that appears as an apparent speeding of the macroscopic time course of inactivation and is nonselective among HVA channels (Roullet *et al.*, 1999). The latter effect shows a striking dependence on carbon chain length (with the highest affinity occurring with 12 or 13 carbons), and is dramatically enhanced by the presence of an amine group at the tail end (Beedle and Zamponi, 2000). The authors proposed that the charged nitrogen group serves to anchor the drug to the channel, while the flexible carbon tail end may ultimately mediate the blocking action. To date, the exact site of action on the N-type channel molecule remains unknown, and it remains to be determined as to whether farnesol analogues will ultimately yield clinically useful inhibitors of N-type calcium channels.

The anticonvulsant gabapentin (1-(aminomethyl) cyclohexaneacetic acid) blocks calcium influx through N-, L-, and P/Q-type calcium channels (Martin *et al.*, 2002; McClelland *et al.*, 2000; Meder and Dooley, 2000; Sutton *et al.*, 2002), possibly through direct interactions with the α_2-δ subunit (Field *et al.*, 2000; Gee *et al.*, 1996). However, direct electrophysiological evidence with recombinant channels is still lacking. Moreover, it remains to be determined if the reported action of gabapentin as an analgesic is indeed related to its inhibition of voltage-gated calcium channels.

Finally, with an IC_{50} in the low micromolar range (Arnoult *et al.*, 1998; Cribbs *et al.*, 1998), mibefradil was originally thought to be a T-channel-specific blocker and was used to treat hypertension, ischemia, and heart failure (Ertel and Ertel, 1997; Mulder *et al.*,1997, 1998; Roux *et al.*, 1996; Vacher *et al.*, 1996). However, further studies have since shown that mibefradil has similar affinities for HVA calcium channels (Bernatchez *et al.*, 2001; Bezprozvanny and Tsien, 1995; Viana *et al.*, 1997). In addition, mibefradil blocks potassium currents (Chouabe *et al.*, 2000; Perchenet and Clement-Chomienne, 2000); block of ATP-activated potassium channels occurs with an IC_{50} less than that for T-type calcium channels (Gomora *et al.*, 1999). Thus, it is no longer appropriate to consider this compound to be selective for T-type currents.

FINAL REMARKS

Most calcium channels contain multiple target sites for antagonists-binding sites, including binding sites for naturally occurring toxins, small organic compounds, and

for metal ions. Among the former two classes, selective antagonists for P-type, N-type, and L-type calcium channels have been identified, but no truly specific blocker of T-type calcium channels has been discovered despite exhaustive efforts. L-type calcium channels are, to date, the only channels for which numerous specific small organic blockers are known. With increasing structural information and sophisticated molecular modeling, we may soon be in the position to rationally design selective calcium channel antagonists, and to, perhaps, identify novel drug target sites on these fundamentally important channel molecules.

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