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Molecular Basis of Drug Interaction with L-Type Ca^{2+} Channels

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Different types of voltage-gated Ca^{2+} channels exist in the plasma membrane of electrically excitable cells. By controlling depolarization-induced Ca^{2+} entry into cells they serve important physiological functions, such as excitation-contraction coupling, neurotransmitter and hormone secretion, and neuronal plasticity. Their function is fine-tuned by a variety of modulators, such as enzymes and G-proteins. Block of so-called L-type Ca^{2+} channels by drugs is exploited as a therapeutic principle to treat cardiovascular disorders, such as hypertension. More recently, block of so-called non-L-type Ca^{2+} channels was found to exert therapeutic effects in the treatment of severe pain and ischemic stroke. As the subunits of different Ca^{2+} channel types have been cloned, the modulatory sites for enzymes, G-proteins, and drugs can now be determined using molecular engineering and heterologous expression. Here we summarize recent work that has allowed us to determine the sites of action of L-type Ca^{2+} channel modulators. Together with previous biochemical, electrophysiological, and drug binding data these results provide exciting insight into the molecular pharmacology of this voltage-gated Ca^{2+} channel family.

KEY WORDS: Calcium channel blockers; drug binding domains; dihydropyridines; ion channels; mutagenesis.

HISTORICAL ASPECTS

More than 20 years ago the German Physiologist Albrecht Fleckenstein discovered that a number of chemically unrelated drugs (Fig. 1A), such as nifedipine (a dihydropyridine, DHP) and verapamil (a phenylalkylamine, PAA) can mimic the effects of simple Ca^{2+} withdrawal on isolated and intact mammalian myocardium by inhibiting depolarization-induced Ca^{2+} ion influx (Fleckenstein, 1983). From these studies the concept of Ca^{2+} antagonism as a new pharmacodynamic principle arose and these drugs were termed Ca^{2+} antagonists. The molecular basis of Ca^{2+} antagonism is the reversible block of plasmalemmal L-type Ca^{2+} channels (LTCCs). Therefore the term Ca^{2+} channel blockers (CCBs) is more appropriate. Since many years CCBs represent a major therapeutic principle in the treatment of cardiovascular diseases like hypertension and angina pectoris.

In the early 1980's the first reports appeared describing saturable high- and low-affinity binding of tritiated DHPs to, e.g., heart muscle and brain membranes (Bellemann *et al.*, 1981; Glossmann *et al.*, 1982; Gould *et al.*, 1982). The low-affinity binding components were unrelated to voltage-gated Ca^{2+} channels and later turned out to represent interesting CCB binding structures such as a nucleoside transporter (Striessnig *et al.*, 1985), sigma1 receptors (Moebius *et al.*, 1997), or a sterol isomerase (Moebius *et al.*, 1994). As expected, the high-affinity binding component represented a stereoselective interaction domain for DHPs on LTCCs. The discovery that LTCCs were expressed at an about 50-fold higher density in skeletal muscle

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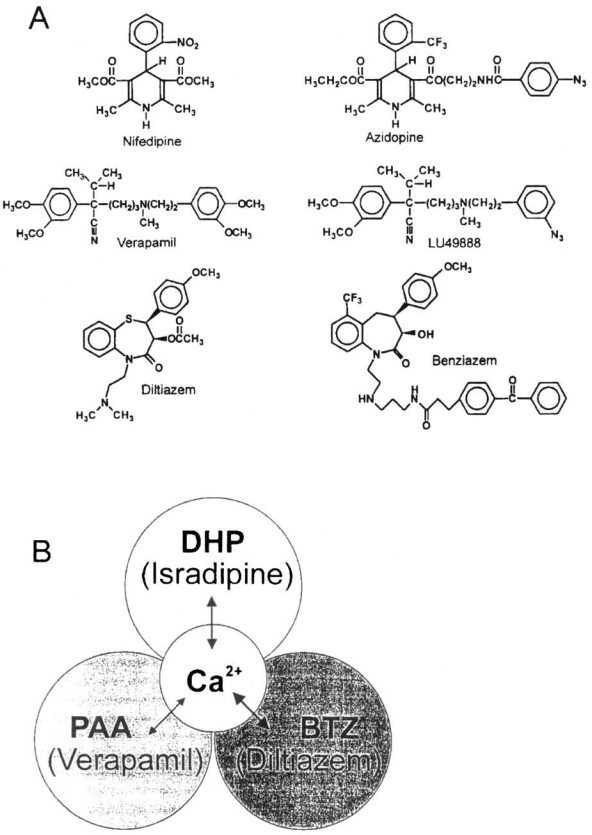

Fig. 1. (A) Chemical structure of clinically important Ca^{2+} antagonists (left) and their photoreactive derivatives (right) employed for photoaffinity labeling. (B) Allosteric model: The bi-directional arrows symbolize allosteric interactions between individual drug binding domains (circles) and Ca^{2+} binding sites of the channel.

compared to vascular smooth muscle and heart (for review see Glossmann and Striessnig, 1988) set the stage for the purification (Glossmann *et al.*, 1983; Curtis and Catterall, 1984) and subsequent cloning of the LTCC subunits (Tanabe *et al.*, 1987). Once the primary structure of the α1 subunit that carries the high-affinity drug-binding domains was known, the identification of the amino acids involved in drug binding was only a matter of time. In this article we review the methodology successfully employed for this purpose and discuss the molecular basis of the Ca^{2+}-sensitive allosteric binding of Ca^{2+} channel active drugs to LTCCs.

THE ALLOSTERIC MODEL

With high-affinity tritiated probes available from the three major chemical classes of CCBs, their molecular pharmacology could be investigated. Equilibrium and kinetic binding analysis indicated that DHPs, PAAs, and a third class of CCBs, the benzothiazepines (BTZs), were able to affect each other's binding, even though they seemed to interact with distinct sites on the channel. For example, DHP binding was noncompetitively inhibited by the PAA verapamil but stimulated by the BTZ (+)-*cis*-diltiazem (Glossmann *et al.*, 1983). These findings were summarized in a model (Fig. 1B) that postulated allosteric interactions between a DHP-, a PAA-, and a BTZ-selective domain[3] (*allosteric model*). Drug interaction with these domains was also affected by cations, such as Ca^{2+}. When brain membranes were treated with EDTA, high-affinity DHP binding time-dependently disappeared but could be restored by addition of micromolar concentrations of free Ca^{2+} or other divalent cations (Glossmann *et al.*, 1985). Similar results were obtained for PAA binding to partially purified skeletal muscle Ca^{2+} channels (Knaus *et al.*, 1992). Higher (millimolar) concentrations of divalent cations inhibited drug binding to all domains. Therefore one or more Ca^{2+} binding sites allosterically coupled to the drug binding domains were included into the *allosteric model* (Glossmann *et al.*, 1985). These reversible binding studies provided a preliminary, mechanistic insight into the molecular organization of these domains. However, many of the observed noncompetitive binding interactions remained phenomenological and awaited detailed structural information for their interpretation (see below).

PHOTOAFFINITY LABELING AND ANTIBODY MAPPING

A first important step toward this goal was the identification of the subunit responsible for drug binding. This was accomplished in photoaffinity and affinity labeling experiments. As outlined in accompanying articles of this minireview series, LTCCs exist as a hetero-oligomeric complex of an α1 (200 kDa), an α2δ (about 170 kDa), a β (60 kDa), and a γ (33 kDa) subunit. In cardiac, smooth, and skeletal muscle membranes some of the (photo)affinity labeling experiments identified 32–45-kDa (for review see

[3] Instead of drug receptors we prefer the term drug-binding domain. Pharmacological classification reserves the term receptor for proteins mediating endogenous agonist effects.

Glossmann and Striessnig, 1990) polypeptides. As shown later these were not associated with Ca^{2+} channels and represented low-affinity, high-capacity sites. In membranes and purified Ca^{2+} channel preparations the arylazide [^{3}H]azidopine specifically labeled 155–190-kDa polypeptides that corresponded to Ca^{2+} channel $\alpha 1$ subunits in skeletal muscle (Ferry *et al.*, 1984), heart (Ferry *et al.*, 1987), and brain (Striessnig *et al.*, 1988). Subsequently, PAA and BTZ photoaffinity probes revealed that the drug-binding domains for these two classes of compounds were also located on the $\alpha 1$ subunit (Striessnig *et al.*, 1987; Striessnig *et al.*, 1990b; Naito *et al.*, 1989).

The photoreactive side chains of these probes were assumed to form covalent bonds either with residues of the drug interaction domain or at least with amino acids that are located in close proximity. To delineate these domains photoaffinity-labeled skeletal muscle LTCC preparations were proteolytically digested and the position of the resulting photolabeled peptide fragments determined within the $\alpha 1$ subunit. The two methodological approaches employed were either gas-phase sequencing of HPLC-purified fragments or antibody mapping. The first approach had been successfully employed to, e.g., localize photolabeled amino acid residues within nicotinic acetylcholine receptors (Dennis *et al.*, 1988). It provides high structural resolution (at the level of photolabeled amino acids) but requires picomolar amounts of HPLC-purified, photolabeled peptides for sequencing. Although this method proved very useful when applied to surface-exposed binding pockets for hydrophilic ligands such as acetylcholine, it does not allow the efficient recovery of peptides containing highly hydrophobic sequence stretches. Moreover, arylazides form acid-labile covalent bonds that are susceptible to cleavage at the low pH used during reversed-phase HPLC separation (Regulla *et al.*, 1991).

The antibody mapping approach involves the immunoprecipitation of defined photolabeled proteolytic fragments with a panel of sequence-directed antibodies at neutral pH. In the presence of detergent even hydrophobic peptides can be reliably immunoprecipitated. The structural resolution of this approach is, however, limited by the size of the proteolytic labeled fragment. This method, which has initially been used to map contact regions for iodinated alpha scorpion toxin on the α subunit of voltage-gated Na^+ channels (Tejedor and Catterall, 1988), accomplished the localization of the drug binding domains within the Ca^{2+} channel $\alpha 1$ subunit. The general experimental strategy for mapping of the BTZ binding domain is illustrated in Fig. 2. The smallest tryptic fragments could be assigned to sequence stretches containing the transmembrane segments S6 in repeats III and IV. The same regions were previously labeled by the DHPs azidopine, diazipine, and isradipine (Nakayama *et al.*,

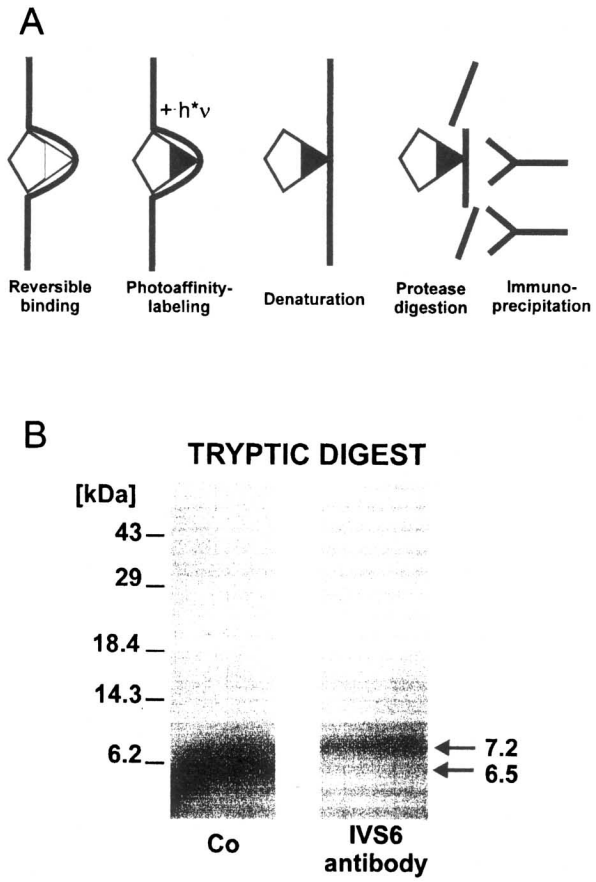

Fig. 2. Antibody mapping of benziazem photolabeled regions: The $\alpha 1S$ subunits of purified skeletal muscle Ca^{2+} channels were specifically photoaffinity labeled with benziazem. (A) To determine the site(s) of photolabeling, labeled $\alpha 1$ subunits were denatured, digested with trypsin, subjected to antibody mapping, and immunoprecipitated peptides visualized by fluorography. (B) Two tryptic fragments (6.5 and 7.2 kDa) were specifically photolabeled (Co). Lane 2 shows that the 7.2-kDa but not the 6.5-kDa fragment was specifically immunoprecipitated by a sequence-directed antibody recognizing an epitope on the cytoplasmic end of segment IVS6 (IVS6 antibody). The same fragment was also recognized by an antibody against an epitope on the extracellular end of IVS6. Therefore the labeled fragment must contain IVS6 together with adjacent extracellular and intracellular amino acid residues. Analogous experiments revealed that the 6.5-kDa fragment contains transmembrane segment IIIS6 (Kraus *et al.*, 1996). Antibody mapping studies with DHPs and PAAs were carried out using the same approach (for review see Catterall and Striessnig, 1992). Data are taken from Kraus *et al.* (1996).

1991; Striessnig *et al.*, 1991), whereas LU49888, a photoreactive PAA (Fig 1A), incorporated only into a fragment that contained transmembrane segment IVS6 (Striessnig *et al.*, 1990a) (Fig. 3A). S6 segments of voltage-gated cation channels are believed to be arranged close to the center of the molecule, thus participating in the formation of the pore (Durell and Guy, 1997). Therefore the mapping results suggested that the drug-binding domains are located close to the ion-conducting pathway of the α1 subunit. The position of photolabeled tryptic peptides in α1 subunits is illustrated in Fig. 3A.

MUTATIONAL ANALYSIS

As cDNA clones encoding the α1 subunits of CCB-sensitive (L-type) and CCB-insensitive (non-L-type) Ca^{2+} channels were isolated, mutational analysis could be employed as a potent tool to confirm the photoaffinity labeling data and to obtain a higher resolution of the drug-binding domains. Genetic engineering was used for the construction of chimeric and mutant α1 subunits. After heterologous expression in mammalian cells or *Xenopus* oocytes, drug sensitivity of the recombinant proteins was monitored in radioligand binding studies or by means of electrophysiological techniques. Two different but complementary approaches were applied.

Gain of Function

Mutational analysis of drug-binding sites usually involves a so-called *loss-of-function* approach in which mutations of one or several amino acid residues within a functional domain are usually expected to decrease biological function, such as enzymatic activity or ligand binding. A typical example is *alanine-scanning mutagenesis* in which amino acid residues are systematically replaced by alanine (Cunningham and Wells, 1989). On the other hand, *gain-of-function* approaches have been increasingly used to characterize ligand–receptor interactions on the molecular level. In this experimental strategy mutational changes are designed to evoke a defined biological function in a protein that lacks the property of interest, such as drug sensitivity. This can be achieved by transferring or creating a distinct drug interaction domain. For example, amino acid residues participating in binding of the angiotensin receptor antagonist losartan were identified by reconstituting losartan sensitivity in a (losartan-insensitive) receptor isoform (Ji *et al.*, 1995). Separate agonist and peptide antagonist binding sites of the oxytocin receptor were identified after transfer of oxytocin receptor sequence into the related vasopressin receptor (Postina *et al.*, 1996). It also allowed the identification of amino acids conferring binding affinity for delta-opioid receptors in a delta/mu-opioid receptor chimera

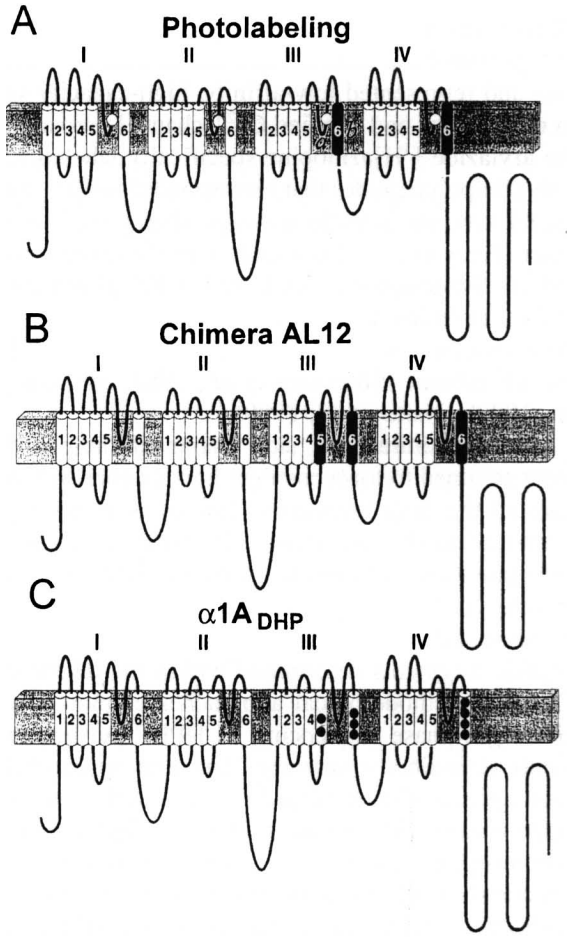

Fig. 3. Drug binding regions within the L-type Ca^{2+} channel α1 subunits. The proposed folding structure is based on hydropathy analysis and structural information obtained on homologous subunits of voltage-gated K^+ - and Na^+ channels. (A) Position of photoaffinity-labeled tryptic peptides. Photoincorporation of photoreactive derivatives was as follows: DHPs: all three fragments, LU49888 (PAA): fragment c; benziazem (BTZ derivative): fragments b and c. White circles indicate the conserved glutamate residues forming the selectivity filter. (B) Structure of the DHP-sensitive Ca^{2+} channel α1 chimera AL12. L-type sequence (gray) was introduced into the corresponding regions of α1A subunits thereby transferring DHP agonist and antagonist sensitivity. (C) Full DHP sensitivity could even be transferred by only mutating nine α1A amino acid residues to L-type, creating mutant $\alpha 1A_{DHP}$. The approximate position of these residues is indicated by dots.

(Pepin *et al.*, 1997). The approach has also been extended to voltage-gated cation channels for the identification of a scorpion toxin interaction site on Kv1.3 potassium channels (Gross *et al.*, 1995).

In the case of LTCCs Grabner *et al.* (1996) used the DHP-insensitive α1A Ca^{2+} channel subunit as an acceptor to investigate if the transfer of the previously photolabeled L-type α1 sequence stretches can evoke DHP sensitivity. α1A is the pore-forming subunit of P/Q-type Ca^{2+} channels, which, together with N- and R-type Ca^{2+} channels, represent the family of high-voltage activated DHP-insensitive non-L-type channels. Replacement of α1A sequence by L-type (α1C, α1S) sequence in regions corresponding to the photolabeled peptides (Fig. 3A) did not yield a DHP sensitive chimera. However, when the L-type transmembrane segment IIIS5, which was not photolabeled by any of the photoreactive DHPs, was added, the resulting construct (termed AL12, Fig. 3B, Grabner *et al.*, 1996) showed all characteristics of high-affinity modulation by DHP agonists and antagonists. It was concluded that the photolabeled sequence stretches were indeed required, albeit not sufficient, for DHP modulation.

The findings of this study also suggested that, at least in skeletal muscle α1S, the S5–S6 linkers in repeats III and IV to some extent contribute to DHP sensitivity (Grabner *et al.*, 1996; Peterson *et al.*, 1996). However, as outlined below, the most crucial DHP interaction sites seem to be located in the transmembrane segments.

Analogous chimeras containing L-type sequence of the PAA photolabeled IVS6 segments were also constructed and found to be clearly sensitive to PAAs (Döring *et al.*, 1996). Taken together these findings suggest that, in the case of Ca^{2+} channel α1 subunits photolabeling was a potent method to pinpoint drug-interaction domains even though it did not reveal all sequence stretches involved in their formation (such as IIIS5 for DHPs and IIIS6 for PAAs, see below).

The importance of IIIS5 was confirmed by the systematic analysis of a DHP-insensitive chimeric α1 subunit. It allowed us to pinpoint the L-type amino acid residues responsible for DHP interaction in transmembrane segment IIIS5. Out of nine nonconserved amino acids that could possibly account for the difference in DHP sensitivity between L-type and non-L-type α1 IIIS5 segments, only Thr-1039 and Gln-1043,[4] which face the same side of a putative alpha helix (Fig. 4), support DHP sensitivity (Mitterdorfer *et al.*, 1996). In agreement with this finding, mutation of these residues in L-type α1C to the corresponding

[4] For simplicity all L-type amino acid residues are given for α1C-II, genebank accession number M67515.

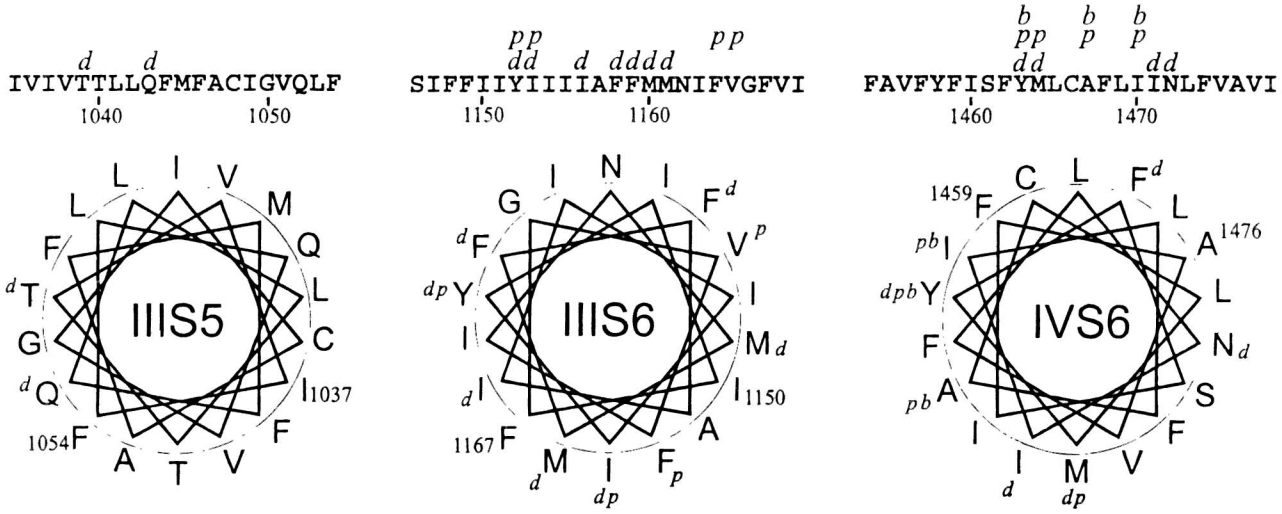

Fig. 4. Amino acid residues that participate in the formation of the DHP, PAA, and BTZ binding domain. Amino acid sequence is given in single letter code. Numbering is according to α1C-II (Genebank accession number M67515). Lower case letters indicate the contribution of single amino acid residues to DHP (*d*), PAA (*p*), and BTZ (*b*) binding. The position of these residues in transmembrane segments IIIS5, IIIS6, and IVS6 is depicted in the primary sequence (upper panel) and in the helical wheel plots which indicate their orientation. Most residues face one side in an alpha helix. Exceptions suggest that these segments may not fold as ideal helices.

α1A residues dramatically decreased DHP affinity in functional and binding experiments (He *et al.*, 1997).

Loss of Function

Amino acids responsible for DHP or PAA interaction in transmembrane segments IIIS6 and IVS6 were identified by systematic replacement with alanine or non-L-type α1 (α1E, α1B) sequence.

Dihydropyridines

In addition to the two IIIS5 residues, at least 11 additional amino acids, seven in IIIS6 and four in IVS6, participate in the formation of the binding pocket for DHPs. The relative contribution to binding affinity in α1C (Peterson *et al.*, 1996; Peterson *et al.*, 1997; Schuster *et al.*, 1996) is most pronounced for Tyr-1152 (IIIS6), Ile-1156 (IIIS6), Met-1161 (IIIS6), and Asn-1472 (IVS6). Four of the 13 residues are conserved in L-type and non-L-type α1 subunits. This suggests that a part of the DHP interaction domain already exists in non-LTCCs but is not sufficient for high-affinity DHP sensitivity. All amino acids known to contribute to DHP binding so far are summarized in Fig. 4.

Phenylalkylamines and Benzothiazepines

The PAA binding pocket is composed of at least seven amino acid residues. In segment IVS6 the L-type residues Tyr-1463, Ala-1467, Ile-1470 (Hockerman *et al.*, 1995; Hering *et al.*, 1996), and Met-1464 (Hering *et al.*, 1996) contribute to high-affinity PAA interaction. Tyr-1152, Ile-1153, Phe-1164, and Val-1165 in IIIS6 (Hockerman *et al.*, 1997a) participate in PAA binding even though these residues are entirely conserved throughout L-type and non-L-type α1 subunits. Two conserved glutamates in repeats III and IV that are part of the Ca^{2+} selectivity filter (see below) also seem to contribute binding affinity for PAAs, presumably by serving as acidic acceptors for the basic nitrogen (Hockerman *et al.*, 1997b) (Fig. 1B).

Hering *et al.* (1996) restored the PAA binding pocket in the α1A subunit (which is very weakly PAA-sensitive due to the conserved IIIS6 residues) by introducing L-type amino acid residues into segment IVS6. The resulting mutant α1A subunit displayed PAA sensitivity comparable to the L-type α1C subunit. Most interestingly, BTZ sensitivity was also restored in this mutant (Hering *et al.*, 1996), suggesting that the three IVS6 residues are also important for BTZ sensitivity, in agreement with photolabeling data (see above and Fig. 2). This indicates that these binding domains at least partially overlap or might be even identical. The PAA site also shares common residues with the DHP site (e.g., Tyr-1152, Ile-1153, Tyr-1463, and Met-1464). This emphasizes the close proximity of all three drug-binding domains.

A DHP-Sensitive α1A Subunit

Chimera AL12 (Grabner *et al.*, 1996) clearly demonstrated that the transfer of extended L-type sequence portions from α1C or α1S into non-L-type α1 subunits allows the transfer of DHP sensitivity. But would a small number of residues, i.e., nonconserved residues identified in the above studies, also be sufficient? This question was addressed by independent studies using α1A (Sinnegger *et al.*, 1997; Hockerman *et al.*, 1997d) and α1E (Ito *et al.*, 1997) as the molecular substrate. Sinnegger *et al.* (1997) found that full DHP sensitivity could be transferred to the α1A subunit after introduction of nine nonconserved L-type amino acid residues in IIIS5, IIIS6, and IVS6 as illustrated in Fig. 3C. The resulting mutant, $\alpha 1A_{DHP}$, displayed all biophysical hallmarks of L-type channel modulation by DHP antagonists (e.g., shift of the steady-state inactivation curve to hyperpolarized potentials) and agonists (e.g., shift of voltage-dependent activation to more hyperpolarized potentials, slowing of deactivation). Besides confirming the importance of these L-type residues for DHP interaction, this study also indicated that the folding geometry of the α1A pore region is very similar to L-type α1 subunits, thus arranging the molecular determinants of DHP interaction in an appropriate distance to form a fully functional DHP binding domain in an α1A background.

INTERACTION OF DRUG AND Ca^{2+} BINDING

As outlined above, Ca^{2+} coordination with the channel is tightly coupled to drug binding. Early binding experiments with radiolabeled DHPs revealed that Ca^{2+} ions must occupy a high-affinity Ca^{2+} binding site in order to stabilize high-affinity DHP binding. Treatment of brain and cardiac Ca^{2+} channels with

EDTA induced a rapid conversion of the DHP binding domain to a low-affinity state. The conversion back to the high-affinity state was achieved by micromolar Ca^{2+} concentrations (Glossmann *et al.*, 1985).

Electrophysiological experiments also predicted a high-affinity Ca^{2+} binding site on the channel since Ca^{2+} channels are about 1000 times more selective for divalent cations than for, e.g., Na^+. Even though Na^+ currents through the channel could be observed in the absence of extracellular divalent cations, these were blocked by micromolar concentrations of Ca^{2+} added to the extracellular solution (for review see Tsien *et al.*, 1987). This was explained by selective Ca^{2+} binding to the channel, thus blocking Na^+ current without giving rise to substantial Ca^{2+} conductance at these low concentration. Obviously, selectivity is achieved by the selective binding of divalent cations before permeation. The selectivity filter has recently been identified on the Ca^{2+} channel $\alpha 1$ subunit. It is composed of four glutamate residues in the S5–S6 linkers of each repeat that are conserved throughout all Ca^{2+} channel $\alpha 1$ subunits. These residues are believed to be asymmetrically arranged around the extracellular mouth of the ion pore (Yang *et al.*, 1993; Tang *et al.*, 1993). Charge neutralization by mutation of one or more of these glutamates to glutamine decreased the channel's affinity for Ca^{2+} ions in electrophysiological experiments and thus its divalent cation selectivity (Yang *et al.*, 1993; Tang *et al.*, 1993). This finding prompted studies which tested the prediction that the selectivity filter also represents the Ca^{2+} binding site responsible for the stabilization of DHP binding. Therefore analogous mutations were introduced into the cardiac $\alpha 1C$ subunit and their effects were tested on DHP binding after heterologous expression in mammalian cells (Mitterdorfer *et al.*, 1995). Individual mutation of any of the four glutamates in $\alpha 1C$ caused a dramatic reduction in DHP ((+)-[^{3}H]isradipine) binding activity which was most pronounced for glutamate mutations in repeats III (EIIIQ) and repeat IV (EIVQ) (Fig. 5A). Kinetic analysis of the EIQ and EIIQ mutants revealed that EDTA more rapidly induced the low-affinity DHP binding state. This is compatible with the prediction that Ca^{2+}, which is required for stabilization of DHP binding, was more loosely bound to these mutants. Interestingly, (+)-tetrandrine, a Ca^{2+} antagonist that binds to the BTZ binding domain (Felix *et al.*, 1992) could stabilize Ca^{2+} dissociation and partially recover DHP binding activity (Fig. 5). This drug effect was not observed in EIIIQ and EIVQ mutants, suggesting that Ca^{2+} and/or (+)-tetrandrine interaction with either of these glutamates is a prerequisite for the stabilization of the DHP binding domain. These experiments could clearly demonstrate that not only DHPs but also drugs interacting with other binding domains affect Ca^{2+} coordination at the selectivity filter (Mitterdorfer *et al.*, 1995). It is therefore conceivable that improved Ca^{2+} binding to the selectivity filter is the underlying mechanism leading to channel block by these drugs.

A more quantitative analysis of the mutational effects on DHP binding was possible by introducing the corresponding mutations into a skeletal muscle $\alpha 1S$ subunit (Peterson and Catterall, 1995). When coexpressed with auxiliary subunits in mammalian cells Ca^{2+} stabilized (stimulated) DHP binding to $\alpha 1S$ subunits in a concentration-dependent manner with an EC_{50} of about 0.6 μM. The concentration–effect curves were shifted toward higher EC_{50} values by the individual glutamate mutations, suggesting a decrease in Ca^{2+} affinity. The largest effects were seen for EIIIQ and EIVQ (11–35-fold) (Peterson and Catterall, 1995). As in brain membranes (Glossmann *et al.*, 1985), the effects of Ca^{2+} on DHP binding were biphasic with binding inhibition occurring at higher (millimolar) concentrations. These data were quantitatively analyzed by a model that assumes high-affinity binding of one Ca^{2+} ion (stabilizing DHP binding) and simultaneous low-affinity binding of two Ca^{2+} ions (inhibiting DHP binding). This model is especially attractive because it attempts to link the biochemical data with the current biophysical explanation for Ca^{2+}-selective permeation. Ca^{2+} (and other divalent cations) can achieve high flux rates through the channel pore despite their high affinity (micromolar K_d) for the selectivity filter. In conducting channels the binding of a second Ca^{2+} ion destabilizes the complex and thereby enables permeation. If a single Ca^{2+} ion stabilizes DHP binding, then the resulting ternary complex between Ca^{2+}, the DHP molecule, and the DHP binding site of the pore-forming $\alpha 1$ subunit should in turn stabilize binding of a single Ca^{2+} ion to the selectivity filter. The model would predict that this complex is equivalent to a nonpermeating, inactivated state, thus explaining block by DHPs. Although the available data (Peterson and Catterall, 1995; Mitterdorfer *et al.*, 1995) suggest that channel modulation by DHPs (and possibly other Ca^{2+} channel active drugs) may be accomplished by improving Ca^{2+} coordination at the selectivity filter, several important questions remain to be answered. First, it is still unknown whether the molecular mechanism of the Ca^{2+} effect on DHP binding results from a conformational change, a direct interaction of the DHP with the bound ion(s) (Zhorov and

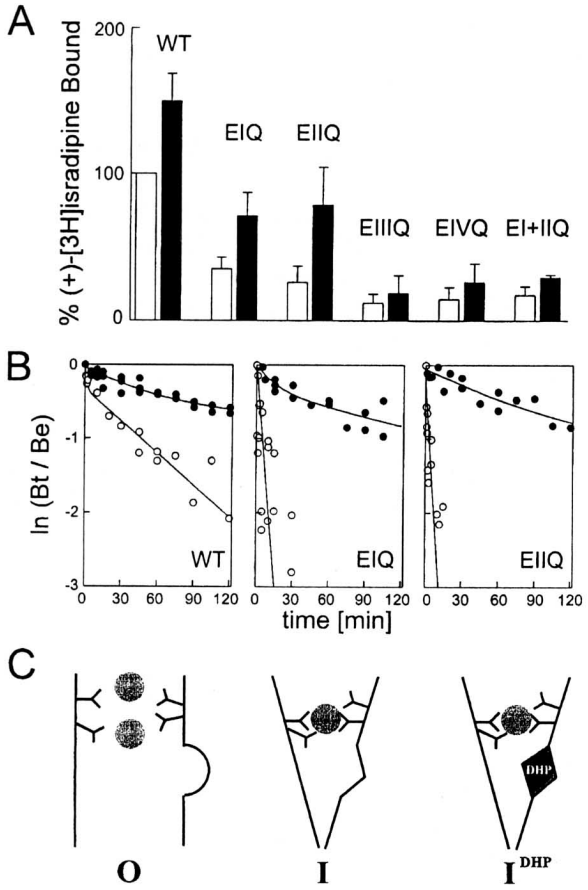

Fig. 5. Mutations in the Ca^{2+} channel selectivity filter decrease the affinity of DHP and Ca^{2+} binding. (A) DHP equilibrium binding activity of wild-type and mutant α_1 subunits at 1 nM (+)-[^{3}H]isradipine and 1 mM Ca^{2+} in the absence (open bars) and presence (filled bars) of 3 μM (+)-tetrandrine. Data were normalized for each experiment to control binding of wild-type membranes in the absence of (+)-tetrandrine (=100%). Data were determined in triplicate and error bars indicate the S.D. of at least three separate transfections. (B) Time course of EDTA-induced conversion reaction to a low-affinity DHP binding state monitored for membranes obtained from COS-7 cells transfected with wild-type α_{1c} (left panel), EIQ (middle panel), and EIIQ (right panel). After 60–120 min incubation with (+) -[^{3}H]isradipine in the absence (open circles) and presence (filled circles) of 3 μM (+)-tetrandrine the reduction of total Ca^{2+} from 1 mM to ~10 nM was initiated by the addition of excess EDTA. Specifically bound radioligand was measured at the times indicated. The solid lines represent logarithmic transformations of the curves obtained by nonlinear curve fitting to a mono- or biexponential decay. Each data point was measured in triplicate. *Bt* is specifically bound ligand at time *t*, and *Be* is specifically bound ligand at equilibrium ($t = 0$). Auxiliary β_{1a} subunits were cotransfected with wild-type and mutant α_1 subunits in all experiments. Data are taken from Mitterdorfer *et al.*(1995). (C) Illustration of the conclusions drawn from the model of interdependence between DHP and Ca^{2+} binding. The α_1 subunit is schematically drawn in the open (left panel), inactivated (middle panel), and inactivated, DHP-blocked (right panel) state. The model of interdependence between Ca^{2+} and DHP binding assumes that in conducting channels (open state, *O*) two Ca^{2+} ions (light gray circles) are loosely coordinated at the glutamate residues (*Y*) of the selectivity filter, rendering the DHP binding domain in a low-affinity state (semi-circular notch). In the inactivated, nonconducting state (*I*, middle panel) a single Ca^{2+} ion is tightly coordinated at the selectivity filter, and the DHP binding domain assumes a high-affinity DHP binding conformation (triangular notch). In this conformation (I^{DHP}, right panel) the DHP molecule (gray diamond) forms a ternary complex with its binding domain and the single Ca^{2+} ion at the selectivity filter. This complex is assumed to stabilize the inactivated state of the channel.

Ananthanarayanan, 1997), or both. Second, from electrophysiological experiments it is clear that DHPs preferentially interact with the inactivated state of the channel (Bean, 1984). The above model predicts that in this state a single Ca^{2+} is bound with high affinity. This hypothesis would provide a link between biochemical and biophysical findings, but it also suggests an interdependence between voltage-dependent inactivation and Ca^{2+} binding to the selectivity filter. To date there is no obvious experimental evidence (e.g., from markedly altered inactivation properties of P-region glutamate mutants (Yang *et al.*, 1993; Tang *et al.*, 1993) supporting this assumption. Only Ca^{2+}-dependent inactivation has been suggested to involve the selectivity filter (Zong *et al.*, 1994). Therefore further functional experiments are required to test this model.

BETA SUBUNITS STABILIZE Ca^{2+} AND DRUG BINDING TO THE α1 SUBUNIT

As outlined in other reviews of this series the auxiliary β, α2-δ, and γ subunits stabilize α1 subunit function. The most important subunit in this regard is the β subunit. When co-expressed with α1 subunits in *Xenopus* oocytes or mammalian cells β subunits change the biophysical properties of α1. A great number of sometimes seemingly conflicting reports suggested a variety of effects, some of which are discussed in Hofmann *et al.* (1994), Perez-Reyes and Schneider (1995), and Isom and Catterall (1994). For example, β subunits alter voltage-dependent gating of the α1 subunit (e.g., Lacerda *et al.*, 1991) increase the number of channels available for opening upon depolarization (e.g., Neely *et al.*, 1993) and affect α1-mediated channel modulation by G-proteins (e.g., Roche *et al.*, 1995).

Several laboratories reported that the co-expression of β subunits dramatically enhanced L-type α1-

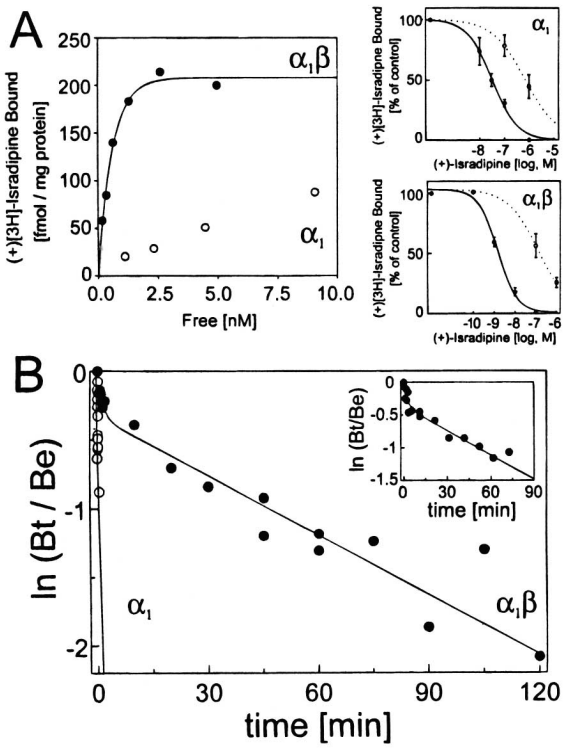

Fig. 6. Co-expression of the β subunit increases the affinity of DHP and Ca^{2+} binding sites of the α1 subunit. (A) Left panel: Saturation isotherm (solid line) of (+)-[^{3}H]isradipine binding to membranes from COS-7 cells transfected with the α1C subunit alone (open circles) or in combination with the β1a subunit (filled circles). K_d and B_{max} values for the β1a cotransfected cells, calculated after Scatchard transformation (r = 0.95), were 0.69 nM and 256 fmol/mg of protein, respectively. Data points represent the mean of three independent determinations. The right panels illustrate the stereospecific inhibition of (+)-[^{3}H]isradipine binding to membranes from COS-7 cells transfected with the α1C subunit alone (upper right panel) or in combination with the β1a subunit (lower right panel) by (+)-isradipine (filled circles) and (−)-isradipine (open circles). Data were obtained in triplicate and normalized to control binding (= 100%). Error bars indicate the S.D. from three independent transfections. The K_i values, obtained after fitting the data to the general dose response equation, were 34.3 ± 6.9 nM (nH = 0.8) for α1C and 0.9 ± 0.2 nM 8 (nH = 1.0) for α1C plus β1a. (B) Time course of EDTA-induced conversion reaction to a low-affinity DHP binding state monitored for membranes obtained from COS-7 cells transfected with α1C alone (open circles) or in combination with β1a (filled circles). After 60 min incubation with (+)-[^{3}H]isradipine the reduction of total Ca^{2+} to ~10 nM was initiated by the addition of excess EDTA and specifically bound radioligand was measured at the times indicated. The conversion rate constant was 2.16 min^{-1} when α_{1C} was expressed alone. Co-expression of the β subunit resulted in a biphasic conversion reaction with rate constants of 0.73 and 0.014 min^{-1} for the fast (27%) and the slow (73%) component, respectively. The inset shows data obtained with guinea-pig cardiac membranes. A similar, biphasic conversion reaction with rate constants of 0.43 min^{-1} (35%) and 0.014 min^{-1} (65%) for EDTA-induced decay of (+)-[^{3}H]isradipine binding was observed. The solid lines represent logarithmic transformations of the curves obtained by nonlinear curve fitting. Each data point was measured in triplicate. Data are taken from Mitterdorfer *et al.* (1994).

associated high-affinity DHP binding (Lacerda *et al.*, 1991; Castellano *et al.*, 1993; Perez-Garcia *et al.*, 1995; Wei *et al.*, 1997; Mitterdorfer *et al.*, 1994). This is not necessarily due to a β subunit-mediated increase in α1 subunit expression or β subunit-mediated translocation (Chien *et al.*, 1995) of channel complexes to the plasma membrane. Instead, β subunit association seems to cause a conformational change of α1 that, similar to the action of Ca^{2+}, converts the DHP binding domain to a high-affinity state. If the low-affinity (β subunit deficient) state remains undetected in radioligand binding analysis, it is expected that β subunit co-expression leads to an apparent increase in B_{max} in saturation studies (Lacerda *et al.*, 1991; Perez-Garcia *et al.*, 1995). On the other hand, if the low-affinity state can be detected, the result is a decrease in K_d without major changes in B_{max} (Mitterdorfer *et al.*, 1994). An example is illustrated in Fig. 6. Binding of the DHP (+)-[^{3}H]isradipine was measured in membranes prepared from COS-7 cells transfected with the cardiac α1C in the absence or presence of β1a. Specific and stereoselective binding was detectable in both membrane preparations. However, in β1a cotransfected cells the affinity for (+)-[^{3}H]isradipine was increased about 35-fold. This conversion from low- to high-affinity DHP binding is reminiscent of the effect of Ca^{2+} ions as discussed above. As illustrated in Fig. 6B, β subunits slow EDTA-induced Ca^{2+} dissociation from α1, suggesting that they also stabilize Ca^{2+} binding to the selectivity filter. The combined effects on Ca^{2+} and DHP interaction suggest that β subunits stabilize the pore region of the channel that contains these binding sites.

DRUG ACCESS TO Ca^{2+} ANTAGONIST BINDING DOMAINS

The available structural information suggests that the drug binding domains are located close to the ion conduction pathway within the plane of the plasma membrane. Thus they are likely not to be exposed on the surface of the α1 subunit. It is therefore not obvious whether drug access can occur from both sides of the channel or only from either

the extracellular or the intracellular side. This question has been studied in electrophysiological experiments using quaternary derivatives of the different chemical classes of Ca^{2+} antagonists. These compounds are membrane-impermeable and can quickly access their binding site only via hydrophilic accession pathways from the side of application. The first domain to be studied was the PAA binding domain. A quaternary derivative of methoxyverapamil, D890, only blocked Ca^{2+} channels in ventricular myocytes when injected into the cell or application through the recording pipette but not after application to the perfusion medium (Hescheler *et al.*, 1982). This finding has been confirmed by many laboratories in a variety of different muscle cells, suggesting that an external barrier exists for PAA access to its site (see references in Berjukov *et al.*, 1996, but see Wegener and Nawrath, 1995). Using the same approach, the opposite result was found for quaternary DHPs (Kass *et al.*, 1991; Strübing *et al.*, 1993) and BTZs (Hering *et al.*, 1993). These compounds only block Ca^{2+} channels after extracellular application, indicating that the drug is unable to access its receptor from the cytoplasm. It is at present unclear which kind of barrier (steric, electrostatic) is responsible for the restriction of drug access. Local anaesthetic drugs block voltage-gated Na^+ channels in a manner very similar to Ca^{2+} channel block by PAAs. Block is more pronounced at higher stimulation frequency (use-dependent), access for quaternary derivatives is limited to the cytoplasmic side of the cell (Hille, 1991; Ragsdale *et al.*, 1994), and, like a part of the PAA interaction site, the amino acid residues responsible for local anaesthetic binding are located on one side of the IVS6 helix of the Na^+ channel α subunit (Ragsdale *et al.*, 1994). Interestingly, the sidedness of drug access to the local anaesthetic binding domain could be modified by mutation of a single amino acid. Replacement of Ile-1760 in the rat brain type IIA Na^+ channel by alanine allowed a quaternary compound to block the channel from the extracellular side, suggesting that an extracellular accession pathway was established by this mutation (Ragsdale *et al.*, 1994). This finding indicates that steric hindrance prevents drug access from the extracellular side in the wildtype Na^+ channel. Similar experiments need to be carried out in LTCC $\alpha 1$ subunit mutants to reveal the structural barriers determining drug access.

STERIC INTERACTIONS BETWEEN Ca^{2+} ANTAGONISTS

In summary, these structural data suggest that all three chemical classes of Ca^{2+} antagonists bind to the same region of L-type $\alpha 1$ subunits and, in part, even use identical residues for interaction. How can these structural findings explain the observed noncompetitive interaction between the different drug-binding domains? If we assume drug-induced conformational changes (allosteric interactions) then these are likely to be short range in nature and may be even limited to the pore region of the channel where drug binding seems to occur. However, based on the close proximity of the binding domains, steric interactions between simultaneously bound drug molecules could also account for at least some of the noncompetitive binding interactions. Evidence for a direct interaction between DHP and BTZ compounds has recently been presented (Brauns *et al.*, 1997). DHPs are known to stabilize BTZ binding (e.g., of (+)-*cis*-[^{3}H] diltiazem) after formation of a ternary complex (Glossmann *et al.*, 1983). To more closely investigate this phenomenon a fluorescent diltiazem analogue, DMBODIPY-BAZ, was synthesized and used to develop a novel binding assay based on fluorescent resonance energy transfer (FRET) (Brauns *et al.*, 1995). In this assay DMBODIPY-BAZ was not excited directly at 488 nm but instead by radiationless energy transfer from tryptophan residues of the channel protein excited at 285 nm. This energy transfer between the energy acceptor (the DMBODIPY fluorophore) and the donor (tryptophan) only occurs within close distance and requires tight ligand binding to the protein. As a consequence the fluorescent signal is mainly derived from specifically bound but not from free ligand. Changes in the concentration of bound ligand can be monitored in real time, without the necessity to separate bound from free ligand (i.e., without disturbing binding equilibrium) (Brauns *et al.*, 1997). In detailed kinetic studies it was shown that the enantiomers of the DHP isradipine decreased association and dissociation kinetics of DMBODIPY-BAZ in a concentration-dependent manner.

The effect was saturable with maximal slowing of association kinetics (see Fig. 7A) at micromolar concentrations of (+)-isradipine. These kinetic changes occurred without changes in K_d and equilibrium binding (Brauns *et al.*, 1997). In addition, DHP association caused a second change in DMBODIPY-BAZ fluorescence that was not associated with a

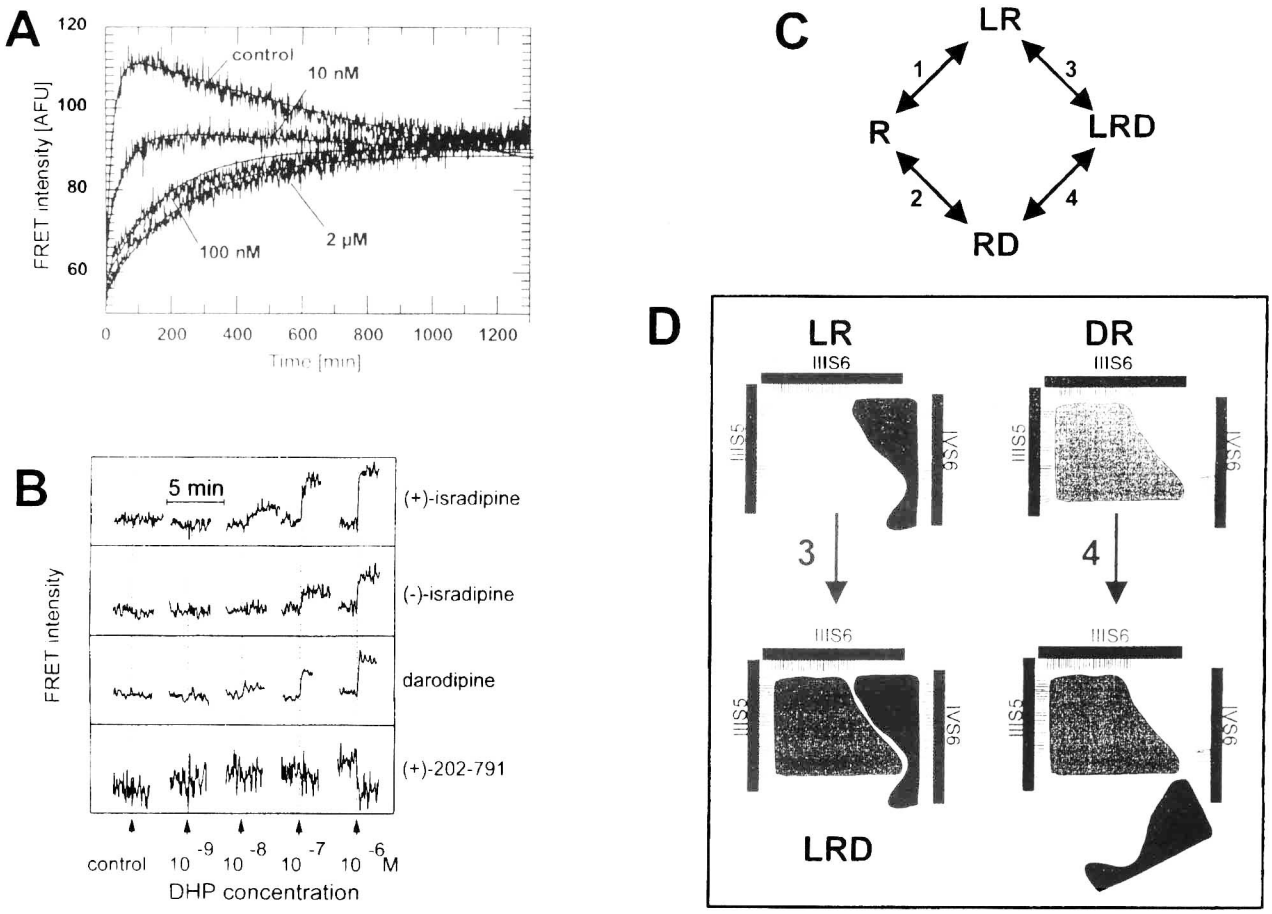

Fig. 7. Analysis of the noncompetitive binding interaction between DHPs and BTZs using fluorescence resonance energy transfer (FRET). (A) Association reaction of DMBODIPY-BAZ to partially purified skeletal muscle LTCCs as determined in real time by FRET. Association was measured in the absence (control) and presence of 10, 100, and 2000 nM (+)-isradipine. The fluorescent signal is given in arbitrary units. Excitation 488 nm; emission 517 nm (for experimental details see Brauns *et al.* 1997). (B) DHPs cause an increase or decrease of DMBODIPY-BAZ fluorescence. DHPs were added to the DMBODIPY-BAZ-channel complex after binding equilibrium had been obtained as in panel A. The indicated DHPs were added at the indicated concentrations and the resulting fluorescence change was measured. These rapid fluorescence changes did not affect DMBODIPY-BAZ binding as determined in separate binding experiments. (C) Reaction scheme describing the binding interaction between DHPs and DMBODIPY-BAZ. L, DMBODIYP-BAZ; D, DHP; R, channel protein. The binding reactions for DMBODIPY-BAZ are fast if no DHP is bound (reaction 1; formation of LR). After DHP binding the accession pathway for the ligand is (partially) occluded. Therefore the binding and unbinding reaction of the ligand are slow in the presence of DHP (reaction 4; formation of LRD). Simultaneous fitting of the experimental data shown in panel A revealed that the bound DHP decreased the association rate constant of DMBODIPY-BAZ by 25-fold. (D) Possible interaction between DMBODIPY-BAZ and (+)-isradipine within the *multisubsite domain* compatible with the reaction scheme in panel C. The DHP (gray) recruits most of its binding energy by interacting with sites in segments IIIS5 and IIIS6 but also forms contacts within IVS6. In contrast, DMBODIPY-BAZ (black) seems to interact preferentially with segments IIIS6 and IVS6. Binding of the DHP prohibits fast association of DMBODIPY-BAZ. Note that in IVS6 Tyr-1463 participates in the binding of both drugs. This implies that DMBODIPY-BAZ can decrease DHP binding affinity. The quantitative analysis as discussed in panel C revealed the expected decrease ($K_{d3}/K_{d2} = 3.3$) and was confirmed in radioligand binding experiments (R. L. Kraus, unpublished). Data are taken from Brauns *et al.* (1997).

change of ligand binding (Fig. 7B). Addition of DHPs to specifically bound (but not free) DMBODIPY-BAZ at binding equilibrium caused a concentration-dependent and rapid change in the fluorescence signal. The direction of this intensity modulation depended on the chemical composition of the DHP employed. (+)-, (−)-isradipine and darodipine increased the signal, whereas other structurally highly related DHPs containing a nitro group, such as the enantiomers of 202-791, nitrendipine and niguldipine, were inhibitory. The effect on fluorescence was independent on the intrinsic agonist and antagonist activity of the compound, mak-

ing it very unlikely that conformational alterations of the DMBODIPY-DHP–α1 complex were responsible for this phenomenon. Instead it was explained on the basis of a direct drug–drug interaction, because nitro groups are known fluorescence quenchers (Lakowicz, 1983). A model that could account for both the kinetic changes and direct fluorescent effects was therefore proposed (Brauns *et al.*, 1997). It predicts that binding of the DHP molecule occurs within the accession pathway of DMBODIPY-BAZ close to but outside its binding site. Therefore DMBODIPY-BAZ association and dissociation is slowed by the DHP through steric hindrance without an effect on equilibrium K_d but close enough to affect its fluorescent properties. A simple reaction scheme describing this steric interaction is illustrated in Fig. 7C. After accounting for the enhanced fluorescent yield of the ternary complex (L–R–D in Fig. 7C) it quantitatively described the experimental data (for parameters see legend to Fig. 7C and Brauns *et al.*, 1997). A hypothetical binding scheme that also implicates some of the available structural details is illustrated in Fig. 7D.

HOT SPOTS FOR DRUG BINDING AND ACTION ON VOLTAGE-GATED ION CHANNELS

Based on these results obtained with photoaffinity labeling, site-directed mutagenesis, and novel fluorescent Ca^{2+} antagonists the early *allosteric model* has to be modified. Rather than distinguishing separate binding domains on the channel we now propose that the pore region of the channel functions as a unique hot spot for drug binding and action where interaction sites for different chemical classes of drugs are formed by adjacent amino acids and to some extent even overlap. As shown for DHPs this hot spot can also be created in non-LTCC subunits. The Ca^{2+} channel α1 subunit is pseudosymmetric. It consists of four homologous repeats surrounding a central ion pore, which is partially lined by the S6 segments as illustrated in Fig. 8A. From the analysis of the DHP binding domain we propose that S5 segments may also be located nearby (Fig. 8A). In the case of DHPs, PAAs, and BTZs the S6 segments in repeats III and IV clearly provide the major portion of binding energy. However, there is no obvious reason to exclude the S6 and S5 segments of repeats I and II from this hot spot. This view is supported by the finding that in the α subunit of voltage-gated Na^+ channels, segments IS6 form part

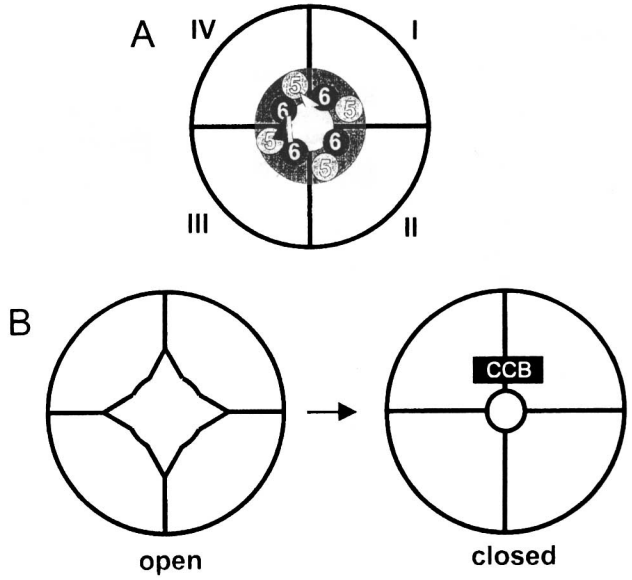

Fig. 8. Multisubsite domain and domain interface model. (A) Binding of CCBs to LTCC α1 subunits can be explained on the basis of a *multisubsite domain model*. This model assumes the existence of a pore-associated hot spot (gray inner circle) that binds different chemical classes of drugs in close vicinity and, in some cases, even with partially overlapping subsites. This hot spot seems to comprise at least the S5 and S6 segments in the four repeats as indicated. The approximate positions of a bound DHP (black triangle), PAA (white square), and brevetoxin (Na^+ channel, white triangle) are indicated. (B) On the basis of this model, drug action can be explained by a *domain interface model*. This model predicts that Ca^{2+} channel modulation by drugs requires the binding of the drug in a domain interface of the channel, such as the interface formed by two adjacent repeats. The *domain interface domain model* is drawn according to Catterall and Striessnig (1992).

of the binding domains for the nonpeptidergic toxins brevetoxin (together with IVS5) and batrachotoxin, respectively (Trainer *et al.*, 1994, 1996). It is tempting to speculate that the four-fold symmetry and the amphiphilic sequence environment of the pore region can provide a high density of suitable attachment sites (for, e.g., hydrogen bond formation as well as hydrophobic interactions) for different drugs. This can not only explain why several different chemical classes of drugs can bind at the same time but also account for the observation that two molecules of the same compound can simultaneously associate with their binding site (as discussed for (+)-*cis*-diltiazem in Prinz and Striessnig, 1993).

In contrast to the early *allosteric model*, we therefore propose an *amphiphilic multisubsite domain* that assumes the existence of a single pore-associated site, accommodating different chemical classes of drugs (Fig. 8A). One of the predictions of our model is that

this region also contains the sites for other known classes of high-affinity CCBs such as HOE166, diphenylbutylpiperidines, butyrophenones, indolizine sulfones, amiloride analogues (for review see Glossmann and Striessnig, 1990), and mibefradil (Bezprozvanny and Tsien 1995). Detailed mapping of their interaction domains should challenge this hypothesis.

Sites with the properties of the *multisubsite domain*, i.e., relatively broad specificity combined with seemingly allosteric binding interactions, also exist on other proteins. Examples are enzymes in the endoplasmic reticulum, such as the emopamil binding protein (a mammalian sterol isomerase involved in a late step of cholesterol synthesis; see Moebius *et al.*, 1997) and sigma 1-receptors (a mammalian homologue of a yeast sterol isomerase; see Moebius *et al.*, 1997). P-glycoprotein, a mammalian plasma membrane drug transport ATPase, recognizes and transports a large number of different drugs including certain DHPs (such as the photoaffinity labels BZDC-DHP and azidopine) and the PAA verapamil (Boer *et al.*, 1996). Very similar to LTCCs, allosterically coupled binding domains selective for, e.g., vinca-alkaloids and DHPs were postulated (Ferry *et al.*, 1992; Boer *et al.*, 1996). It is conceivable that drug interaction with all these proteins is also restricted to hot spots that may be associated with specialized functional domains (sterol binding and catalysis in the case of isomerases, and sterol, phospholipid, and xenobiotic binding and transport in the case of P-glycoprotein; see Debry *et al.*, 1997).

How can drug binding affect channel function? The structural data clearly demonstrate that binding of DHPs, PAAs, and BTZs involves more than one hydrophobic repeat (e.g., S6 helices in repeats III and IV). This observation led Catterall to propose a *domain interface model* (Catterall and Striessnig, 1992; Hockerman *et al.*, 1997b) for drug action implying that modulation by CCBs requires binding in the interface of two separate structural domains of the channel (Fig. 8B). This model is based on the finding that modulatory regions on enzymes are also located preferentially at domain interfaces. DHPs, PAAs, and BTZs do not simply act by plugging the channel pore. Instead, they interfere with channel gating. It is therefore likely that drug binding at a domain interface within the *multisubsite domain* allows the drug to confer its modulatory effects to pore-associated gating structures. The exact molecular mechanisms underlying channel modulation by CCBs are not understood and may vary for different drugs. Direct or indirect interference with Ca^{2+} coordination has been discussed above as a possible mechanism. Another hypothesis assumes that a direct interaction of the S6 segments is involved in the voltage-dependent process of Ca^{2+} channel inactivation gating (Hering *et al.*, 1996). This hypothesis is supported by the finding that mutations within these segments have pronounced effects on Ca^{2+} channel inactivation (Zhang *et al.*, 1994; Peterson *et al.*, 1997; Hering *et al.*, 1997). Based on the available data, the functional consequences of drug binding could therefore also be interpreted in terms of altered S6 segment interactions.

CONCLUSIONS

By elucidating the molecular basis of drug interaction with LTCCs novel concepts must be devised to account for the biochemical and functional data initiated by Fleckenstein's research. At present our concept of an *amphiphilic multisubsite domain* nicely accounts for drug binding and a *domain interface model* for drug action. Further research will challenge these concepts and thus provide a more detailed understanding about the mechanism of drug action on voltage-gated cation channels.

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