Benzothiazepine Ca\(^{2+}\) antagonists (such as (+)-cis-diltiazem) interact with transmembrane segments IIIS6 and IVS6 in the \(\alpha_1\) subunit of L-type Ca\(^{2+}\) channels. We investigated the contribution of individual IIIS6 amino acid residues for diltiazem sensitivity by employing alanine scanning mutagenesis in a benzothiazepine-sensitive \(\alpha_1\) subunit chimera (AL\(_{DIL}\)) expressed in Xenopus laevis oocytes.

The most dramatic decrease of block by 100 \(\mu\)M diltiazem (AL\(_{DIL}\) 45 \(\pm\) 4.8\% inhibition) during trains with 100-ms pulses (0.1 Hz, 
\(-80\) mV holding potential) was found after mutation of adjacent IIIS6 residues Phe\(_{1164}\) (21 \(\pm\) 3\%) and Val\(_{1165}\) (8.5 \(\pm\) 1.4\%). Diltiazem delayed current recovery by promoting a slowly recovering current component. This effect was similar in AL\(_{DIL}\) and P1164A but largely prevented in V1165A. Both mutations slowed inactivation kinetics during a pulse. The reduced diltiazem block can therefore be explained by slowing of inactivation kinetics (P1164A and V1165A) and accelerated recovery from drug block (V1165A). The bulkier diltiazem derivative benziazem still efficiently blocked V1165A.

From these functional and from additional radioligand binding studies with the dihydropyridine (+)-[\(^{3}H\)isradipine we propose a model in which Val\(_{1165}\) controls dissociation of the bound diltiazem molecule, and where bulky substituents on the basic nitrogen of diltiazem protrude toward the adjacent dihydropyridine binding domain.

The activity of voltage-gated L-type Ca\(^{2+}\) channels in muscle, endocrine and neuronal cells is blocked by different chemical classes of drugs, termed Ca\(^{2+}\) antagonists. Dihydropyridines (nifedipine, isradipine), phenylalkylamines (verapamil, desmethoxyverapamil) and benzothiazepines ((+)-cis-diltiazem; diltiazem\(^{3}\)) stereoselectively interact with high affinity binding domains on the so-called \(\alpha_1\) subunit of the L-type Ca\(^{2+}\) channel complex and thereby cause channel block (1).

Recent biochemical studies, employing photoaffinity labeling, site-directed mutagenesis, and chimeric \(\alpha_1\) subunit constructs revealed that high affinity binding determinants for these drugs are located close to pore-forming regions of L-type Ca\(^{2+}\) channel \(\alpha_1\) subunits. Important binding determinants for DHPs and PAAs were identified in transmembrane segments IIIS5, IIIS6, and IVS6 (for review, see Refs. 2 and 3). Both classes of drugs directly or indirectly affect Ca\(^{2+}\) coordination to the channel's selectivity filter glutamates. It is therefore conceivable that these drugs cause Ca\(^{2+}\) channel block by binding to pore-forming regions, thereby altering channel gating and Ca\(^{2+}\) ion interaction with the pore (for review, see Refs. 2 and 3).

Less detailed structural information is available for the BTZ binding domain, which mediates use-dependent Ca\(^{2+}\) channel block by diltiazem. BTZ and PAA interaction with the channel is similar in many respects; BTZs and PAAs contain a basic nitrogen essential for activity (4) and, unlike for DHPs, Ca\(^{2+}\) channel block critically depends on depolarization frequency ("use-dependent block"). Some early radioligand binding experiments even proposed that PAAs and BTZs compete for the same site (5) on the channel. This is supported by the finding that identical amino acid residues in IVS6 are essential for high affinity PAA as well as diltiazem sensitivity (2, 6). On the other hand several lines of evidence suggest that the PAA and BTZ binding domains are not identical; PAAs and BTZs possess a distinct structure-activity relationship for blocking (4) and they access their binding domains from opposite sides (PAAs, the cytoplasmic side; BTZs, the extracellular side) of the channel (7).

In an extensive analysis combining information from alanine-scanning mutagenesis and radioligand binding we provide further insight into the molecular architecture of the BTZ binding domain of L-type Ca\(^{2+}\) channels. In particular we identified two amino acid residues in transmembrane segment IIIS6 which affect diltiazem sensitivity by different mechanisms and provide evidence for a steric interaction between the BTZ and DHP binding domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—The (+)-cis-Disatereocisem of diltiazem was employed in all experiments (kindly provided by Göddeke (Freiburg, Germany). (+)-Isradipine was a gift from Sandoz AG (Basel, Switzerland). (+)-[\(^{3}H\)Isradipine (\(-80\) Ci/mmol) was purchased from New England Nuclear (Vienna, Austria). Benziazem was synthesized as described previously (8).

**Mutant \(\alpha_1\) cDNAs, Alanine Scanning Mutagenesis**—The diltiazem-sensitive \(\alpha_1\) subunit chimera AL\(_{DIL}\) was employed for alanine scanning mutagenesis. This chimera (Fig. 1) consists of a1A sequence with an

\[
\text{hydroxy-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2H-1-benzazepine-2-one;}
\]

\(I_{\text{Ba}}\), barium inward current; PAA, phenylalkylamine.
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L-type Ca^{2+} channel sequence transferred into the S5-S6 linkers as well as S6 segments in III and IV.

Chimera ALDIL (amino acid composition: α1A1-1400-α1S905-1104 (carp skeletal muscle; GenBank accession no. M62554), α1A1-1446-α1C1311-1477, rabbit heart; GenBank accession no. 67515), α1A1-1400-α1S905-1104 was coinjected with α1D4 fragment of ALDIL and ligated into the ClaI-XbaI linearized AL1-a (10). Single point mutations were introduced into the S4l (nucleotide position α1S1311)-ClaI (nucleotide position α1S905) cassette of segment IIIIS6 using gene splicing by overlap extension (11). Polymerase chain reaction was performed (35 cycles, 1 min at 94 °C, 30 s at 62 °C, 1.5 min at 72 °C) using proofreading Taq polymerase. Polymerase chain reaction-generated DNA mutations were verified by sequence analysis employing the dyeoxy chain termination method. Single alanine mutations were introduced into the IIIIS6 segment between the following positions (amino acid positions correspond to α1C-II) (12); Phe1146 to Ile1146 and Phe1146 to Phe1146. All constructs were inserted into the polyadenylating transcription plasmid pSPCBI-2.

Expression of ALDIL Mutants in Xenopus laevis Oocytes—Preparation of stage V-VI oocytes from X. laevis, synthesis of capped run-off poly(A)’ cRNA transcripts from XbaI-linearized cDNA templates, and injection of cRNA were described in detail previously (9). α1a cRNAs were coinjected with β1a (13), and α2-δ subunit (14) cRNAs. To exclude effects of endogenous Ca^{2+}-activated Cl− currents on current kinetics, experiments were also carried out in oocytes previously injected with 50–100 nl of a 0.1 M 1,2-bis-(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid solution.

Electrophysiological Recordings—Inward Ba^{2+} currents (I_\text{Ba}) through expressed channel complexes were measured using the two-microelectrode voltage-clamp technique (9, 15). Recordings were carried out at room temperature in a bath solution containing 40 mM Ba(OH)\textsubscript{2}, 40 mM Na\textsubscript{2}EDTA, 10 mM HEPES (adjusted to pH 7.4 with HCl) and had resistances of 0.3–2 megohm. Similar current amplitudes were obtained with mutant and ALDIL subunits. Oocytes expressing peak I_\text{Ba} smaller than 400 nA or larger than 1.6 mA were excluded from analysis. Data analysis and acquisition was performed by using the pClamp software package (version 6.0, Axon Instruments).

Initial “tonic” block (resting state-dependent block) was defined as peak I_\text{Ba} inhibition during the first pulse after 3-min incubation at holding potential in a drug-containing solution as compared with control I_\text{Ba} in the absence of drug.

Use(frequency)-dependent block of I_\text{Ba} was measured during trains of 100-ms test pulses (0.1 Hz) applied from −80 mV to a test potential +10 mV positive to the peak of the current-voltage relationship after a 3-min equilibration period in a drug-containing solution. To estimate the peak I_\text{Ba} decay under control conditions, similar test pulses were applied in the absence of drug. Use-dependent block was expressed as the percent decrease of peak I_\text{Ba} during the last pulse of the train as compared with I_\text{Ba} during the first pulse.

The voltage dependence of activation was determined from I-V curves obtained by step depolarizations from a holding potential of 80 mV to a test potential +10 mV after 10-s steps to various holding potentials. The half-maximal voltage for activation (V_0.5,act) and steady state inactivation (V_0.5,inact) were calculated by fitting the data to the Boltzmann equation.

Recovery of I_\text{Ba} from inactivation was studied using a double-pulse protocol. After a 3-s depolarizing prepulse to +10 mV (holding potential −80 mV) the time course of I_\text{Ba} recovery was determined at −60 mV by applying 300-ms test pulses to +10 mV at various time intervals after the prepulse. Peak I_\text{Ba} was normalized to the peak current amplitude measured during the prepulse. I_\text{Ba} was then allowed to recover during 90 s at −100 mV. This double pulse protocol was repeated individually for each recovery time interval in the same oocyte.

Radioligand Binding Studies—[1H]Iswradipine binding assays were carried out at 37 °C or room temperature in 50 mM Tris-HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride 0.5–1 ml final assay volume (10% v/v) dithiothreitol. The reaction was started by mixing 1 nM (32) [1H]Iswradipine with 400,000 cpm in 1 μl of assay buffer containing 50 mM Tris-HCl, 100 mM NaCl, 1,000 nM isradipine and 10 μM GDP for 5 min at 22 °C (13). Bound ligand was determined by filtration of the assay mixture over GF/C Whatman filters pretreated with 0.25% (v/v) polyethyleneimine for 40 min. Filters were washed three times with ice-cold buffer (20 mM Tris-HCl, pH 7.4) and then counted for radioactivity. Radioligand and protein concentrations as well as incubation conditions are given in the figure legends. To determine radioligand dissociation, (+)N-[1H]Iswradipine was incubated (in the absence or presence of benzamid) with rabbit skeletal muscle membranes until equilibrium was reached (60 min at 22 °C). After a 15-min incubation, the presence of unlabeled benzamid was then added and dissociation followed for the indicated times. Dissociation rate constants were determined as the slope of the regression line from a plot of ln (fractional binding) versus time. Molecular dynamics simulations (25 °C) were performed using the SYBYL software package.

Data Analysis—Nonlinear least square fitting and statistical calculations were performed using Origin (Microcal). Data are given as means ± S.E. for the indicated number of experiments. Student’s unpaired t test was used to calculate statistical significance.

RESULTS

Alanine Scanning Mutagenesis of Segment IIIIS6—We have previously shown that the photoreactive diltiazem analogue [3H]benzamid photoaffinity labels transmembrane segments IVS6 and IIIIS6, suggesting that both segments are in close contact with the bound drug molecule and contain high affinity BTZ-binding determinants (8). We subsequently identified the binding determinants in IVS6 as Tyr1463, Ala1467, and Ile1470 (6). To search for amino acid residues that affect diltiazem sensitivity in segment IIIIS6 we employed alanine-scanning mutagenesis. Mutagenesis was carried out in a diltiazem and PAA-sensitive α1 subunit chimera ALDIL in which the segments corresponding to the photolabeled regions and the respective S5-S6 linkers consisted of L-type sequence, whereas the remaining sequence consisted of a1A sequence (Fig. 1, see “Experimental Procedures”). Mutated channels were coexpressed in X. laevis oocytes together with auxiliary α2-δ and β subunits and the sensitivity of the mutant channels to diltiazem was determined by using the two-microelectrode voltage-clamp technique. Mutants were screened for changes in resting-state and use-dependent block by diltiazem during a train of 15–20 depolarizing pulses (100 ms, 0.1 Hz) from a holding potential of −80 mV as described under “Experimental Procedures.” As shown in Fig. 2, A and B, 100 μM diltiazem blocked about 45 ± 4.8% (n = 8) of the ALDIL-mediated I_\text{Ba}.

A significant (p < 0.01) reduction in total diltiazem block was observed only after mutation of two adjacent residues, Phe1164 and Val1165, near the cytoplasmic end of IIIIS6. Both residues are conserved in L- and non-L-type Ca^{2+} channel α1 subunits. Only 21 ± 3% (n = 12) and 8.5 ± 1.4% (n = 13) of I_\text{Ba} were blocked by 100 μM diltiazem in F1164A and V1165A, respectively. Accordingly, at lower diltiazem concentrations, block was observed for ALDIL, but was almost absent for F1164A and V1165A (10 μM diltiazem; ALDIL, 30 ± 3%, n = 3; F1164A, 6 ± 2%, n = 3; V1165A, 3–5%, range, n = 2).

The extent of total I_\text{Ba} inhibition of the other mutants was not significantly different from ALDIL (Fig. 2A). In mutants F1148A and 11163A, the tonic block component was increased,

![Fig. 1. Structure of chimera ALDIL. Dark segments and bold lines represent L-type Ca^{2+} channel sequences (repeat III; carp skeletal muscle α1 repeat IV; rabbit heart α1) introduced into a1A (white segments, thin lines).](image-url)
and the use-dependent block component significantly decreased compared with that of AL DIL. These residues may therefore comprise minor determinants for diltiazem sensitivity.

Biophysical Properties of F1164A and V1165A—We have recently shown that the sensitivity of L-type Ca\(^{2+}\) channels to the PAA gallopamil is decreased in slowly inactivating Ca\(^{2+}\) channel α1 mutants (10), suggesting that voltage-dependent inactivation is an important determinant of gallopamil sensitivity. We therefore investigated whether mutations F1164A and V1165A alter diltiazem sensitivity indirectly by changing the gating properties of the channel rather than by directly altering drug affinity for its binding domain.

Fig. 3A illustrates that V1165A (V\(_{0.5,\text{inact}}\) = 16 ± 2 mV, n = 11), but not F1164A (V\(_{0.5,\text{inact}}\) = 36 ± 2 mV, n = 5), caused a major (about 16 mV) shift of the V\(_{0.5,\text{inact}}\) of the steady state inactivation curve to more positive potentials (AL DIL: V\(_{0.5,\text{inact}}\) = 32 ± 2 mV, n = 12). At −80 mV, the holding potential selected for the experiments shown in Fig. 2, no differences in steady state inactivation were observed. Therefore it is unlikely that a higher fraction of inactivated channels at −80 mV accounted for the differences in diltiazem sensitivity in the two mutants. F1164A and V1165A also caused a significant shift of current activation (AL DIL: V\(_{0.5,\text{act}}\) = −17.3 ± 1 mV; F1164A V\(_{0.5,\text{act}}\) = −24.7 ± 1.2 mV; V1165A V\(_{0.5,\text{act}}\) = −10.6 ± 1.1 mV; n > 22) and slowed current inactivation during a pulse. I\(_{Ba}\) elicited by 800-ms depolarizations from a holding potential of −80 mV to test potentials +10 mV positive to the peak of the I–V curve were shown in Fig. 3B. Monoeponential fits of the current decay revealed an about 1.5–3-fold slower inactivation of F1164A (τ = 627 ± 118 ms, n = 10) and V1165A (τ = 1286 ± 79 ms, n = 12) than did AL DIL (τ = 447 ± 103 ms, n = 14). Taken together our data reveal that mutation of two conserved residues, Phe\(^{1164}\) and Val\(^{1165}\), on the cytoplasmic end of transmembrane segment IIIIS6, change activation and inactivation gating and are major determinants for use-dependent diltiazem block.

Mechanism of Channel Block by Diltiazem—If inactivation is an important determinant for the development of use-dependent block by diltiazem, then slowing of channel inactivation in the two mutants could explain their lower sensitivity for diltiazem.

To address this question the kinetics of diltiazem block and unblock were studied in more detail. The effect of 10 and 100 μM diltiazem on the rate of current decay was determined during maintained 3-s depolarizations that allowed substantial inactivation even for the more slowly inactivating mutants F1164A and V1165A (Fig. 4A). In AL DIL, current decay during the 3-s test pulse was accelerated by 100 μM but not by 10 μM diltiazem (Fig. 4A), thereby reducing I\(_{Ba}\) at the end of the test pulse to 64 ± 9% of control (n = 4). This effect was comparable in F1164A (57 ± 6% of control, n = 5) and V1165A (68 ± 3% of control, n = 9). The drug-induced increase of the rate of I\(_{Ba}\) decay is characteristic for drugs that block open and/or inactivated channel states during the pulse more potently than do resting channels at −80 mV. Using the extent of I\(_{Ba}\) block by diltiazem at 3 s as a measure, the mutations did not dramatically affect channel block by 100 μM diltiazem at depolarized potentials (+10 mV).

Next we investigated the effect of diltiazem on the time course of recovery of I\(_{Ba}\) from inactivation employing a double pulse protocol. Fig. 4B shows the recovery of I\(_{Ba}\) for AL DIL, F1164A, and V1165A after a 3-s depolarizing conditional prepulse. Recovery was measured at −60 mV by applying 300-ms test pulses to +10 mV various periods of time after the prepulse. Peak I\(_{Ba}\) elicited by the test pulses were normalized with
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Fig. 3. Biophysical properties of ALDIL, F1164A, and V1165A. A, steady-state inactivation of ALDIL (cell R7903c36), F1164A (cell R7904c17), and V1165A (cell R7903c15). Cells were depolarized from a holding potential of −80 mV to the indicated prepulse potentials for 10 s followed by a test pulse to +10 mV. B, inactivation of $I_{Ba}$ through ALDIL, F1164A, and V1165A during an 800-ms test pulse from −80 mV to a test potential +10 mV positive to the peak current of the corresponding I–V curves. Traces were fit to a monoexponential decay yielding the following time constants ($\tau$, in seconds): ALDIL, 0.405; F1164A, 0.64; V1165A, 1.21. Cells R7911c19 (ALDIL), R7828c34 (F1164A), and R7001c02 (V1165A).

respect to the peak $I_{Ba}$ of the prepulse (see current traces in Fig. 4D). The recovery time courses in the absence of drug were similar for ALDIL and the two mutants (see legend to Fig. 4B). In all cases recovery could be described by two phases. $I_{Ba}$ rapidly recovered monoexponentially to 80–90% of control within 15 s (termed “fast recovery”), whereas the remaining current did not recover within the 15-s period analyzed. From the total recoverable current we arbitrarily defined the contribution of “slow recovery” as the $I_{Ba}$ not recovered after 15 s in the presence of diltiazem (10 $\mu$M diltiazem present: 29 ± 3%, n = 5; 100 $\mu$M diltiazem present: 27 ± 3%, n = 9) was only slightly increased as compared with control (16 ± 1%, n = 9). Diltiazem delayed the time course of the fast recovering component (see legend to Fig. 4B), which resulted in a clear inhibition of $I_{Ba}$ during the first few seconds. To illustrate this difference in current recovery the fraction of current recovered after 3 or 15 s in the presence of 10 $\mu$M diltiazem was divided by the fraction of current recovered in the absence of drug for ALDIL and the two mutants. Fig. 4C illustrates that after 3 s of recovery a comparable percentage of the current remained blocked in all constructs by 10 $\mu$M diltiazem. This degree of block was maintained over 15 s in ALDIL and F1164A but mostly relieved in V1165A (Fig. 4C).

The acceleration of recovery from diltiazem block in mutation V1165A indicates that valine replacement either directly decreases binding affinity for the drug or allows a more rapid dissociation of the drug after being trapped in a blocked channel state. As diltiazem sensitivity in V1165A was still observed as a block of depolarized channels (Fig. 4A) and as a block of $I_{Ba}$ during early recovery (Fig. 4C), mutation V1165A does not seem to dramatically decrease diltiazem binding affinity. Earlier models describing block of L-type Ca$^{2+}$ channels by PAA Ca$^{2+}$ antagonists suggest that use-dependent Ca$^{2+}$ channel blockers bind to open channels, but are trapped within inactivated channels and require removal of inactivation for rapid dissociation and unblocking (10, 16). We therefore propose that replacement of the bulkier valine in position 1165 by an alanine (partially) removes a dissociation barrier and facilitates dissociation of diltiazem from blocked (presumably inactivated) channel states. If this were true then we would expect that bulky diltiazem derivatives would escape at a slower rate. As a consequence such a bulky drug should, at least partially, overcome the effect of the mutation and cause substantial use-dependent block even in V1165A. We tested this hypothesis using the bulkier diltiazem derivative benziazem, which has previously been shown to photofluorophore label segment IIIS6 (8). Benziazem contains a bulky benzophenone substituent remote from the pharmacophores important for interaction with the benzothiazepine binding domain (8). Fig. 5 shows that the extent of block by 10 and 100 $\mu$M benziazem (gray columns) was only slightly (1.3–1.5-fold) larger than by diltiazem in ALDIL. In contrast, under the same experimental conditions benziazem caused 3.6–4.5-fold larger block of V1165A at both concentrations than did diltiazem, indicating that this bulkier drug must still be able to considerably slow recovery from inactivation in V1165A.

Implications for the Molecular Organization of the Drug Binding Domains—The above finding also prompted us to investigate whether the larger size of benziazem can alter its noncompetitive interaction mechanism with the DHP binding domain, which is also formed predominantly by IIIS6 residues (2, 3). We compared the effects of the bulky benziazem and its analogues Bz-BAZ and DMBODIPY-BAZ (18) with the smaller molecules diltiazem and SQ32,910 (7) on (+)-[125I]isradipine binding to skeletal muscle L-type Ca$^{2+}$ channels.
Fig. 4. Effect of diltiazem on current decay during a pulse and on recovery from inactivation. A, $I_{CA}$ was elicited by a 3-s depolarization from a holding potential of −80 mV to a test potential of +10 mV. Representative current traces in the absence (Co) or presence of 10 or 100 μM (+)-cis-diltiazem are shown for ALDIL (cells 97D04000, 97D04001, and 97D04002), F1164A (cells R1009006, R1009007, and R1009008), and V1165A (cells R1009003, R1009004, and R1009005). B, recovery of $I_{CA}$ from inactivation. Peak currents of the test pulses applied various times after the conditioning prepulse (3 s) were normalized to peak currents of the prepulse and plotted against time. Representative experiments are shown. The fast component of the recovery time courses in the absence (Co) or presence of 10 or 100 μM (+)-cis-diltiazem were estimated from a monoexponential fit yielding the following time constants (in seconds): ALDIL, 2.97, 2.27, 3.27 (cell 97D04000, 97D04001, 97D04002); F1164A, 2.29, 2.37, 5.44 (cell R1009006, R1009007, R1009008); V1165A, 1.47, 2.98, 5.28 (cell R1009003, R1009004, R1009005). The following time constants were calculated from three to nine independent experiments: ALDIL, 2.71 ± 0.15, 2.34 ± 0.10, 4.04 ± 0.80; F1164A, 1.93 ± 0.21, 2.87 ± 0.44, 5.83 ± 0.41; V1165A, 1.78 ± 0.17, 3.82 ± 0.43, 6.94 ± 0.77. C, difference in current recovery. The fraction of current recovered after 3 and 15 s in the presence of 10 μM (+)-cis-diltiazem was divided by the fraction recovered in the absence of drug for ALDIL, F1164A and V1165A. Data are means from three to five experiments. D, representative traces of recovery experiments in the presence of 10 μM (+)-cis-diltiazem for ALDIL (cell R1001011) and V1165A (cell R1009004) are shown.

Fig. 5. Concentration-dependent block of ALDIL and V1165A by diltiazem (black columns) and benziazem (gray columns). The pulse protocol was as described in Fig. 2A.

Fig. 6A illustrates that, in contrast to (+)-cis-diltiazem and SQ32,910, which are known stimulators of equilibrium (+)-[3H]isradipine binding (7), all three bulky derivatives were inhibitory. For benziazem and Bz-BAZ incomplete inhibition could be demonstrated (maximal inhibition to 73 ± 3% (n = 10) and 89 ± 2% (n = 3) of control). Benziazem was selected to further investigate the inhibitory mechanism of these compounds. Saturation analysis (Fig. 6B) revealed that 1 μM benziazem decreased the apparent binding affinity of the DHP by about 2.8-fold (control, $K_d = 0.43 ± 0.06$ nM; 1 μM benziazem present, $K_d = 1.2 ± 0.06$ nM; n = 3) without major change in $B_{max}$ (control, 179 ± 22 pm; 1 μM benziazem present, 154 ± 13 pm; n = 3). This affinity decrease was mainly due to a destabilization of the DHP-Ca$^{2+}$ channel complex as revealed by dissociation kinetics. For dissociation experiments (Fig. 6C) (+)-[3H]isradipine was incubated in the absence (control) and presence of 1 μM benziazem until binding equilibrium was reached. (+)-[3H]Isradipine dissociation from control and ternary channel complexes was then induced by an excess of unlabeled isradipine (“cold chase”). As shown in Fig. 6C dissociation was about 2-fold faster from ternary complexes than
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**Fig. 6. Inhibition of (+)-$[^3]$H]isradipine binding to skeletal muscle membranes by bulky diltiazem analogues.** A, modulation of (+)-$[^3]$H]isradipine binding by increasing concentrations of diltiazem and diltiazem analogues. Assay conditions were as follows: radioligand concentrations 0.1–0.4 nM; membrane protein concentration 10–20 μg/ml; incubation time 60 min; incubation temperature: 22 °C or 37 °C ((+-)cis-diltiazem, SQ32,910). The following binding parameters (IC$_{50}$, slope, maximal inhibition) were calculated for inhibitory drugs: benziazem, 20 nM, 0.92, 72%; Bz-BAZ, 6 nM, 0.82, 90%; (+-)verapamil, 72 nM, 0.74, 86.5%; DMBDIPY-BAZ, 60% inhibition at 1 μM. One of at least three similar experiments is shown. Similar binding inhibition was observed after incubation at 37 °C. For binding stimulation by (+)-cis-diltiazem and SQ32,910, see Hering et al. (7). B, saturation analysis. Membrane protein concentration was 10 μg/ml. Binding parameters were calculated by linear regression analysis of the data after Scatchard transformation (inset). Control ( ), $K_D$ = 397 nM, $B_{max}$ = 157 pM; 1 μM benziazem present; ( ) $K_D$ = 1250 pM; $B_{max}$ = 141 pm. C, dissociation kinetics (ligand concentration, 0.26 nM; membrane protein concentration, 10 μg/ml). After reaching (+)-$[^3]$H]isradipine binding equilibrium in the absence or presence of 1 μM benziazem, dissociation from Ca$^{2+}$ channels was induced by the addition of 1 μM (±)-isradipine. The following dissociation rate constants ($k_2$) were obtained from semilogarithmic plots (inset): control ( ), $k_2$ = 0.037 min$^{-1}$; 1 μM benziazem present ( ), $k_2$ = 0.075 min$^{-1}$. Similar results were obtained in an independent experiment (control, $k_2$ = 0.036 min$^{-1}$; 1 μM benziazem present, $k_2$ = 0.064 min$^{-1}$). An almost identical acceleration was found with 10 μM benziazem ($n$ = 3, not shown). D, dissociation kinetics ("double-chase"); ligand concentrations 0.20–0.43 nM; membrane protein concentration, 10–20 μg/ml). After reaching (+)-$[^3]$H]isradipine binding equilibrium in the absence of benziazem, dissociation of (+)-$[^3]$H]isradipine was induced by adding final concentrations of 1 μM (±)-isradipine (control, ); 1 μM (±)-isradipine + 1 μM benziazem ( ), or 1 μM (±)-isradipine + 30 μM verapamil ( ). Control, $k_1$ = 0.041 min$^{-1}$; 1 μM (±)-isradipine + 1 μM benziazem, $k_1$ = 0.043 min$^{-1}$; 1 μM (±)-isradipine + 30 μM verapamil, $k_1$ = 0.081 min$^{-1}$. No significant ($p < 0.01$) increase of $k_1$ by benziazem was found in three independent experiments: Control, $k_1$ = 0.040 ± 0.003 min$^{-1}$; 1 μM (±)-isradipine + 1 μM benziazem, $k_1$ = 0.046 ± 0.004 min$^{-1}$. from channels preincubated without benziazem. This indicated that the increase of $k_1$ mainly accounts for the increase in $K_d$ ($K_d = k_2/k_{-1}$; ($k_{-1}$ = dissociation rate constant; $k_1$ = association rate constant). However, this destabilizing effect of benziazem was not seen when 1 μM (or 10 μM, not shown) benziazem was added (together with unlabeled (+)-isradipine, "double chase") after the (+)-$[^3]$H]isradipine channel complex had already been formed (Fig. 6D). These experiments clearly show that benziazem destabilized DHP binding in the ternary complex but that the DHP must dissociate before this effect can occur. In control experiments (+-)verapamil, which is known to inhibit DHP binding via an allosteric mechanism (1), also partially inhibited (+)-$[^3]$H]isradipine equilibrium binding but, in contrast to benziazem, was also able to accelerate (+)-$[^3]$H]isradipine dissociation from preformed high affinity (+)-$[^3]$H]isradipine-channel complexes (Fig. 6D). The benziazem binding data are difficult to interpret on the basis of an allosteric model, which would predict a destabilizing effect of an unlabeled drug through binding to a separate, allosterically coupled site in both types of dissociation experiments (as observed for (+-)verapamil). Modulation of (+)-$[^3]$H]isradipine binding by benziazem is best explained as a steric interaction between the bound benziazem and DHP molecule, in which the bulky side chain of benziazem can decrease DHP binding stability once the DHP is dissociated. Although a precise mechanism for this effect cannot be derived from our experiments it is likely that this is caused by a protrusion of the bulky benziazem side chain into the DHP binding region.

On the basis of this proposed steric mechanism we can use benziazem and its bulky analogues as molecular rulers. From the distance of their side chains from the basic amine pharmacophore as calculated from molecular dynamics simulations (benziazem ≤ 17 Å; DMBDIPY-BAZ ≤ 17 Å; Bz-BAZ ≤ 11 Å)
we can estimate the maximal distance between the BTZ binding domain and the bound DHP molecule to be 11–17 Å.

**DISCUSSION**

Our study provides insight into the molecular mechanism of diltiazem interaction with L-type Ca$^{2+}$ channels and the molecular organization of the Ca$^{2+}$ antagonist binding domains on $\alpha_1$ subunits. Using alanine scanning mutagenesis we identified amino acid residues Phe$^{1164}$ and Val$^{1165}$ at the cytoplasmic end of the putative IIIIS6 α-helix as important determinants for diltiazem sensitivity. Although our functional studies could not demonstrate their direct contribution to the formation of the diltiazem binding pocket, these residues indirectly control diltiazem sensitivity by slowing channel inactivation (both residues, Fig. 3B) and by facilitating channel recovery from drug block (V1165A, Fig. 4). We also provide evidence that introduction of bulky side chains into the diltiazem molecule (i) largely prevented the V1165A mutational effect by stabilizing channel block and (ii) produced compounds noncompetitively inhibiting rather than stimulating DHP binding to the channel through an apparently steric interaction mechanism.

Among the 19 mutations investigated, mutants F1164A and V1165A caused the most pronounced effects on channel inactivation kinetics as well as diltiazem sensitivity. Our studies do not exclude the possibility that minor effects on diltiazem sensitivity are present in the other mutants which will, however, require a more detailed analysis. Phe$^{1164}$ and Val$^{1165}$ are highly conserved in all high voltage-activated Ca$^{2+}$ channel $\alpha_1$ subunits. As mutation of these residues not only affects inactivation gating in AL$\alpha_1$, but also in $\alpha_1$C (19) it is likely that they are part of a common inactivation mechanism in all Ca$^{2+}$ channel $\alpha_1$ subunits.

Our experiments provide important insight into the mechanism of diltiazem block. It promotes a slowly recovering channel state that explains the development of use-dependent block at a higher depolarization frequency. This effect of diltiazem is qualitatively indistinguishable from the mechanism of action of phenylalkylamines (10, 16). Although different kinetic models have been proposed to account for this effect (10, 16), it is believed that the time course of recovery from channel block is critically determined by steric factors governing the rate of dissociation of the drug trapped within inactivated channel states (10, 16, 20). The intriguing effect of mutation V1165A was to allow the channel to recover from diltiazem block more rapidly. A possible molecular explanation for this finding is that Val$^{1165}$ as part of an inactivation mechanism also controls diltiazem dissociation from inactivated channels. Replacement by a less bulky alanine would therefore not only affect inactivation but also facilitate drug dissociation thereby accelerating recovery from drug block. Such a steric mechanism also predicts that not only the size of the “dissociation pathway” generated by the mutation but also the size of the drug itself affects the rate of dissociation. The fact that the bulkier diltiazem derivative benziazem was still an effective use-dependent blocker of V1165A (Fig. 5) strongly supports the above model. Together with its slower inactivation time course the accelerated recovery from diltiazem block of V1165A also explains the dramatic decrease of its use-dependent block at higher depolarization frequency (Fig. 2).

We describe another interesting difference between diltiazem and its bulkier derivatives benziazem, DBMODYIPY-BAZ, and BZ-BAZ. They act as partial inhibitors rather than stimulators of (+)-[3H]isradipine binding to L-type Ca$^{2+}$ channels. A detailed kinetic analysis of the benziazem effect led us to conclude that partial DHP binding inhibition by the bulky side chains is based on a steric rather than allosteric decrease of (+)-[3H]isradipine binding affinity. Our results are in accordance with our previous detailed spectroscopic analysis of the interaction of fluorescently labeled diltiazem derivative, DM-BODYIPY-BAZ, with unlabeled DHPs (21). We found evidence for a direct drug-drug interaction between channel bound DHPs and DM-BODYIPY-BAZ, also supporting a steric model. From a quantitative analysis of high time resolution kinetic binding data (21) we predicted a 3-fold decrease of (+)-isradipine affinity after DM-BODYIPY-BAZ binding. This is now confirmed directly employing (+)-[3H]isradipine as a radioligand supporting a model in which the DHP and BTZ binding domain are located in close proximity between IIIIS6 and IVS6 in the folded $\alpha_1$ subunit structure. Using the bulky side chains of diltiazem analogues as molecular rulers we currently estimate a maximal distance of 11–17 Å between the basic nitrogen of bound BTZs and the bound DHP molecule. This is about 2 times the narrowest diameter of the channel pore (~6 Å) (22). It will be interesting to investigate the behavior of compounds with even smaller side chains (23) on DHP binding, which may help to get a more precise estimate of the minimal distance between the two bound drug molecules.

Our and previous data reveal several important common properties between PAA and BTZs with respect to channel interaction and modulation. First, they both share residues Tyr$^{1463}$, Ala$^{1467}$, and Ile$^{1470}$ in segment IVS6 as common binding motifs (6, 24); second, Val$^{1165}$ in IIIIS6 affects not only PAA (19) but also BTZ sensitivity (this study); third, despite a clearly distinct structure-activity relationship of PAAs and BTZs both classes of drugs exhibit a similar mechanism of use-dependent block of L-type Ca$^{2+}$ channels, namely by inducing slow recovery from inactivation. Rather than distinguishing between two separate binding domains for PAAs and BTZs as proposed by an allosteric model (1) or strongly overlapping binding domain mostly formed by IIIIS6 and IVS6 residues should be proposed through which cationic amphiphilic PAAs and BTZs modulate L-type channel activity.

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