Identification of Benz(othi)azepine-binding Regions within L-type Calcium Channel α1 Subunits*

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To identify the binding domain for diltiazem-like Ca\textsuperscript{2+} antagonists on L-type Ca\textsuperscript{2+} channel α1 subunits we synthesized the benzazepine [\textsuperscript{3}H]benziazem as a novel photofañinity probe. [\textsuperscript{3}H]Benziazem reversibly labeled the benzothiazepine (BTZ)-binding domain of partially purified skeletal muscle Ca\textsuperscript{2+} channels with high affinity (K\textsubscript{d} = 12 nM) and photoincorporated into its binding domain with high yield (>66%). Antibody mapping of proteolytic labeled fragments revealed specific labeling of regions associated with transmembrane segments S6 in repeats III and IV. More than 50% of the labeling was found in the tryptic fragment alanine 1023-lysine 1077 containing IIIS6 together with extracellular and intracellular amino acid residues. The remaining labeling was identified in a second site comprising segment S6 in repeat IV and adjacent residues. Unlike for dihydropyridines, no labeling was observed in the connecting HIIS5-IIIS6 linker. The [\textsuperscript{3}H]benziazem photolabeled regions must be in close contact to the drug molecule when bound to the channel. We propose that the determinants for high affinity BTZ binding are located within or in close proximity to segments IIIS6 and/or IVS6. Therefore the binding domain for BTZs, like for other main classes of Ca\textsuperscript{2+} antagonists, must be located in close proximity to pore-forming regions of the channel.

Voltage-dependent L-type Ca\textsuperscript{2+} channels are blocked by different groups of chemically unrelated compounds, termed Ca\textsuperscript{2+} antagonists. These drugs cause vasodilation and cardiac depression by blocking L-type Ca\textsuperscript{2+} channels in smooth and cardiac and are used for the therapy of cardiovascular disorders.

One of the clinically most widely used Ca\textsuperscript{2+} antagonist is the benzothiazepine (+)-diltiazem. Like other Ca\textsuperscript{2+} antagonists (e.g., dihydropyridines and phenylalkylamines (1)) it interacts with a high affinity binding domain on the pore-forming α1 subunit of L-type Ca\textsuperscript{2+} channels (2) in heart muscle, brain, and skeletal muscle. The benzothiazepine (BTZ)\textsuperscript{3} selective domain is coupled to the dihydropyridine (DHP)- and phenylalkylamine (PAA)-binding domains via non-competitive mechanisms (1).

To gain deeper insight into the mechanisms of L-type channel modulation by Ca\textsuperscript{2+} antagonists the molecular motifs involved in drug binding must be identified. DHP and PAA interaction sites within the primary structure of L-type α1 subunits were successfully localized by antibody mapping of proteolytic fragments derived from their photoaffinity labeled domains (3-5). Within these regions individual amino acid residues participating in drug interaction were then identified using site-directed mutagenesis (6-9).

As compared to DHPs and PAA, photoaffinity ligands developed for the BTZ-binding domain display low binding affinity and incorporation yields (2, 10, 11). Therefore a complete localization of the BTZ-binding domain using photofañinity labeling and antibody mapping was impossible (10).

We report here the synthesis of a novel photoreactive diltiazem derivative (benziazem) that reversibly labels the BTZ-binding domain of partially purified L-type Ca\textsuperscript{2+} channels with high affinity. Benziazem specifically photoincorporates into the BTZ-binding domain of skeletal muscle α1 subunits with high labeling efficiency. Antibody mapping of photolabeled peptides revealed that the critical determinants of benziazem binding are exclusively localized within regions of α1 that also participate in the formation of the DHP- and PAA-binding domain. Our results suggest that all major classes of Ca\textsuperscript{2+} antagonists bind within the pore-forming regions of repeats III and IV of L-type Ca\textsuperscript{2+} channel α1 subunits.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: l-3-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin, from bovine pancreas), Protein A-Sepharose, and bovine serum albumin (essentially globulin free) from Sigma; endoproteinase Lys-C (Lys-C) from Boehringer Mannheim; prestained molecular weight marker proteins from Bio-Rad (high range) and Life Technologies, Inc. (low range); Amplify from Amersham; digitonin from Biosynth AG (Basel, Switzerland).

Synthesis of [\textsuperscript{3}H]Benziazem—[\textsuperscript{3}H]Benziazem (Fig. 1) was synthesized by coupling N-hydroxy succinimidyl-benzoyl dihydrochymamic acid to the free amino group of a benzazepine precursor ((3R,4R)-cis-1,3,4,5-tetrahydro-6-trifluoromethyl-3-hydroxy-1-[2-[3-aminopropyl]]-2-[1,1-dimethyl ethoxycarbonyl] aminoethyl-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2H-1-benzazepin-2-one; B\textsubscript{max} maximum density of binding sites; Bz-BAZ, (3R,4S)-cis-1-[2-[3-(benzoylamino)propyl]amino]-ethyl-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2H-1-benzazepin-2-one; DHP, dihydropyridine; IC\textsubscript{50}, concentration causing half-maximal inhibition; Lys-C, endoproteinase Lys-C; PAA, phenylalkylamine; PAGE, polyacrylamide gel electrophoresis; TPCK, l-1-tosylamido-2-phenylethyl chloromethyl ketone.

7 nmol of [\textsuperscript{3}H]Benziazem (Fig. 1) was synthesized by coupling N-hydroxy succinimidyl-benzoyl dihydrochymamic acid to the free amino group of a benzazepine precursor ((3R,4R)-cis-1,3,4,5-tetrahydro-6-trifluoromethyl-3-hydroxy-1-[2-[3-aminopropyl]]-2-[1,1-dimethyl ethoxycarbonyl] aminoethyl-4-(4-methoxyphenyl)-2H-1-benzazepin-2-one, Ref. 12). Precursor (3.5 μmol) was suspended in 0.1 ml of 10 mM NaOH and the free base extracted with 3 x 0.1 ml of ethyl acetate. 7 nmol of [\textsuperscript{3}H]hydroxy succinimidyl-benzoyl dihydrochymamic acid (35 Ci/mmol, Du Pont NEN; Ref. 13) were added and the mixture incubated on ice for 10 min. The solvent was evaporated under vacuum and the residue dissolved in 0.1 ml of 0.1 M NaOH and the solution was fractionated through a Bio-Gel P-4 column (1.5 x 20 cm).

The mixture was then applied to a Pharmacia Fast Protein Liquid Chromatography column for separation of the deprotected benzothiazepine (BTZ) from the succinimidyl labeled compound. The recombinant benzothiazepine was obtained from two fractions by preparative HPLC. The specific activity of the benzothiazepine was 22 Ci/mmol. The benzothiazepine was further purified by column chromatography on a Pharmacia FPLC column and the specific activity was measured as 18 Ci/mmol.
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a gentle stream of nitrogen. To remove the Boc protecting group, 0.1 ml of methylene chloride followed by 0.1 ml of trifluoroacetic acid were added and the mixture incubated for 60 min on ice. Methylene chloride/trifluoroacetic acid were removed under vacuum, the reaction products resuspended in 50 μl of ethanol and then separated on a Silica Gel 60 thin layer plate developed in benzene/methanol/diethylether (74/25/1). [3H]Benziazem migrated with an Rf value of 0.57 ± 0.06 (n = 3). After extraction of the radioactive peak into ethanol more than 50% of the applied radioactivity was recovered as [3H]benziazem. Radioactive benziazem was stored in the dark at −25°C.

Sequence-directed Antibodies—Polyclonal anti-a1 antisera were raised in rabbits against synthetic Ca2++ channel peptides corresponding to residues 339–354 (a139–354), 679–692 (a179–92), 1011–1026 (a1101–1026), 1025–1040 (a11025–1040), 1066–1082 (a11066–1082), 1339–1354 (a11339–1354), and 1382–1400 (a11382–1400) of the skeletal muscle α1 subunit (3, 5, 14). The N termini of these peptides are indicated in Fig. 5. For some experiments anti-a11290–1356, anti-a11951–1306, and anti-a11300–1400 were affinity purified on Sepharose 4B derivatized with the corresponding peptides (3, 5). Elution was achieved with 5 mM MgCl2 or 0.1 mM glycine-HCl (pH 2.5). To raise antibodies anti-a1119–309, anti-a111300–1306, and anti-a111025–1040, the connecting SS-S6 linkers of repeats 1, 11, and 1V of the skeletal muscle α1 subunit (14) were expressed as N-terminal fusion proteins in E. coli (strain M15[pREP4]) using expression plasmid pOPE-40 (Qiagen). Fusion proteins were purified by chelate affinity chromatography (Ni-NTA resin, Qiagen) and used for immunization.

Radioisogand Binding Studies—Binding experiments with [3H]benziazem were performed as described (15) in 50 mM Tris-Cl (pH 7.4), 0.1 mM N-phenylmethylsulfonyl fluoride, 0.25 mg/ml bovine serum albumin, 0.1% (v/v) 2-mercaptoethanol in the presence of 1.6% (w/v) digitonin (0.5 ml final assay volume). Nonspecific binding was determined in the presence of 10 μM Bz-BAZ (for chemical structure, see Ref. 12). Incubation conditions for saturation analysis are given in the legend to Fig. 2. For binding inhibition experiments ligand and protein concentrations were 1.2–1.4 nm and 5.6 μg/ml, respectively. After incubation, the assay mixture was cooled on melting ice for 5 min. One ml of bovine serum albumin and γ-globulin were added followed by 4 ml of a solution containing 10% (w/v) polyethylene glycol 6000, 10 mM MgCl2. After precipitation of the labeled complexes for 3 min on ice, free ligand was removed by filtration over GF/C Whatman filters. Filters were washed 5 times with ice-cold buffer (20 mM Tris-Cl, pH 7.4) and then counted for radioactivity.

Preparation of Photolabeled α1 Subunit—Carboxamidomethylation of [3H]benziazem-labeled α1 subunits was achieved by incubation of α1 subunits with iodoacetamide. Freshly prepared iodoacetamide was then added to a final concentration of 0.1 M glycine-HCl (pH 2.5). To raise antibodies anti-a111219–309, anti-a111300–1306, and anti-a111025–1040, the connecting SS-S6 linkers of repeats 1, 11, and 1V of the skeletal muscle α1 subunit (14) were expressed as N-terminal fusion proteins in E. coli (strain M15[pREP4]) using expression plasmid pOPE-40 (Qiagen). Fusion proteins were purified by chelate affinity chromatography (Ni-NTA resin, Qiagen) and used for immunization.

Radioligand Binding Studies—Binding experiments with [3H]benziazem were performed as described (15) in 50 mM Tris-Cl (pH 7.4), 0.1 mM N-phenylmethylsulfonyl fluoride, 0.25 mg/ml bovine serum albumin, 0.1% (v/v) 2-mercaptoethanol in the presence of 1.6% (w/v) digitonin (0.5 ml final assay volume). Nonspecific binding was determined in the presence of 10 μM Bz-BAZ (for chemical structure, see Ref. 12). Incubation conditions for saturation analysis are given in the legend to Fig. 2. For binding inhibition experiments ligand and protein concentrations were 1.2–1.4 nm and 5.6 μg/ml, respectively. After incubation, the assay mixture was cooled on melting ice for 5 min. One ml of bovine serum albumin and γ-globulin were added followed by 4 ml of a solution containing 10% (w/v) polyethylene glycol 6000, 10 mM MgCl2. After precipitation of the labeled complexes for 3 min on ice, free ligand was removed by filtration over GF/C Whatman filters. Filters were washed 5 times with ice-cold buffer (20 mM Tris-Cl, pH 7.4) and then counted for radioactivity.

Preparation of Photolabeled α1 Subunit—Carboxamidomethylation of [3H]benziazem-labeled α1 subunits—Diaalyzed and lyophilized samples were reduced for 15 min at 57°C with 1% (v/v) 2-mercaptoethanol in the presence of 10 μM unlabeled competitor (Bz-BAZ) to determine the specific binding and photoaffinity labeling. Reversible binding prior to photobinding was determined by filtration (15) of 200-μl aliquots of the incubation mixture. Samples were transferred to siliconized glass Petri dishes and irradiated for 90 min at 4°C with ultraviolet light (Philips 38W/TL black light lamp) at a distance of 25 mm. Photolabeled samples were dialogized against deionized water at room temperature for 12 h and lyophilized.

Reductive Carboxamidomethylation of [3H]Benziazem-labeled α1 Subunits—Diaalyzed and lyophilized samples were reduced for 15 min at 57°C with 1% (v/v) 2-mercaptoethanol in the presence of 10 μM unlabeled competitor (Bz-BAZ) to determine the specific binding and photoaffinity labeling. Reversible binding prior to photobinding was determined by filtration (15) of 200-μl aliquots of the incubation mixture. Samples were transferred to siliconized glass Petri dishes and irradiated for 90 min at 4°C with ultraviolet light (Philips 38W/TL black light lamp) at a distance of 25 mm. Photolabeled samples were dialyzed against deionized water at room temperature for 12 h and lyophilized.

Pretrypsin Cleavage—α1 subunits were digested with Lys-C (1.6 units/ml) in 40 mM Tris-Cl (pH 8.5) and 2.5 μM urea for 12 h at 37°C. For TPCK-trypsin digestion, photolabeled α1 subunits were incubated with trypsin (10 μg/ml final concentration) in 60 mM Tris-Cl (pH 8.5) and 0.033% (w/v) SDS for 2 h at 37°C. An aliquot of the digest was removed for direct SDS-PAGE analysis (non-immunoprecipitated control). Digestion was terminated by adding SDS sample buffer. For immunoprecipitation experiments the mixtures were boiled in 0.5% (w/v) SDS for 3 min. The samples were then adjusted to 1% (v/v) Triton X-100, 0.1% (w/v) SDS (or 0.5% (w/v) urea), bovine serum albumin (0.5-1 mg/ml), 150 mM NaCl, and 10 mM Tris-Cl (pH 8.5).

Immunoprecipitation—Sequence directed antibodies were bound to Protein A-Sepharose 4B by incubating 1-3 volumes of serum with 1
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[\textsuperscript{3}H]benziazem were incubated with 5 \(\mu\)M partially purified \(\text{Ca}^{2+}\) channel protein for 90 min at 22°C in the absence (control) and presence (nonspecific binding) of 10 \(\mu\)M Bz-BAZ. Bound ligand was determined by filtration. After photolysis, samples were diluted 10-fold with radioimmunoassay buffer containing 1% Triton X-100 to avoid nonspecific binding (10% was recovered). Specific binding, defined as the percentage of radioactivity was recognized by domain IV antibodies, was found in the 14.2-kDa peak and 38 ± 7 kDa in the larger polypeptides. Immunoprecipitation with sequence-directed antibodies revealed that only antibodies directed against epitopes located near segments S6 in repeats III (anti-\(\alpha_1\)11011–1026, anti-\(\alpha_1\)1025–1040 and anti-\(\alpha_1\)1066–1080) or IV (anti-\(\alpha_1\)1339–1354, anti-\(\alpha_1\)1382–1400 and anti-\(\alpha_1\)1390–1395) immunoprecipitated the photolabeled fragments (Fig. 3B). Other antibodies against the S5-S6 linker in domain I (anti-\(\alpha_1\)1219–309), the cytoplasmic side of S6 (anti-\(\alpha_1\)1339–1354), and the cytoplasmic linker between repeats II and III (anti-\(\alpha_1\)1679–692) efficiently bound \(\alpha_1\) but failed to immunoprecipitate Lys-C fragments (not shown). In at least three independent experiments about 68–74% of the \(\alpha_1\)-associated labeling was associated with fragments recognized by anti-\(\alpha_1\)1011–1026 and anti-\(\alpha_1\)1025–1040 (Fig. 3B). The epitopes of these antibodies are located in the IIIS5–IIIS6 linker and do not contain, nor are they separated by, Lys-C cleavage sites. In the same digest the repeat IV antibodies anti-\(\alpha_1\)1339–1354 and anti-\(\alpha_1\)1382–1400 bound 40–46%. The fact that a smaller percentage of radioactivity was recognized by domain IV antibodies suggested that they were immunoprecipitating the 28.6-kDa band. This was confirmed by SDS-PAGE of antibody-bound radioactivity. Anti-\(\alpha_1\)1382–1400 (Fig. 3, A and C) and anti-\(\alpha_1\)1390–1356 (n = 3, not shown) selectively immunoprecipitated the 28.6- and (when present) 22-kDa fragments. As the intracellular \(\alpha_1\)1382–1400 epitope is located within a single Lys-C fragment that also contains IVS6 and extracellular residues these peptides must represent partial digestion products containing segment IVS6 together with adjacent extra- and intracellular sequence stretches. Anti-\(\alpha_1\)1011–1026 (Fig. 3D) failed to recognize the larger fragments but immunoprecipitated the labeled 14.2-kDa polypeptide. A Lys-C fragment of approximately this mass containing the \(\alpha_1\)1011–1026 epitope must result from cleavage at lysines in positions 962 or 976 and lysines 1077 or 1083 (calculated masses 12–14.5 kDa). Such a fragment contains at least half of the IIIS5–IIIS6 linker, IIIS6 and 12–18 cytoplasmic amino acid residues. Antibody anti-\(\alpha_1\)1066–1080 recognized variable amounts of total specific photolabeling (Fig. 3B) in Lys-C digests. This indicates at least partial cleavage at lysine 1077, resulting in removal of a portion of this antibody’s epitope.

[\textsuperscript{3}H]Beniazem Labeling Is Located in Tryptic Fragments
containing S6 Segments in Repeats III and IV—As the Lys-C fragments also contain trypsin cleavage sites the photolabeled α1 subunits were digested with TPCK-trypsin (10 μg/ml) to further restrict the sites of photolabeling. SDS-PAGE revealed two small photolabeled tryptic fragments with apparent molecular masses of 8.8 ± 0.9 and 7.1 ± 0.5 kDa (n = 4). A fluorogram of a gel where these peaks were clearly separated is shown in Fig. 4A. The α1-associated [3H]benziazem photolabeling was quantitatively recovered (n = 3) in these peaks. In experiments with at least three different α1 preparations, tryptic digests revealed no smaller photolabeled polypeptides (Fig. 4, A and C).

The larger peptide must be derived from the 22–35-kDa Lys-C fragments as it was selectively immunoprecipitated by anti-α1,11011–1040 (Fig. 4, A and C). The other repeat IV antibodies (anti-α1,1139–1354 and anti-α1,1290–1356) also immunoprecipitated radioactivity from the same tryptic digest to a similar extent (Fig. 4D). The labeled peptide must therefore contain IVS6 together with adjacent extracellular and cytoplasmic amino acid residues resulting most likely from cleavage at lysine 1336 and lysines in positions 1403 or 1414 (calculated molecular masses 7.9–9.2 kDa).

In contrast, the 7.1-kDa tryptic fragment was immunoprecipitated by repeat III antibody anti-α1,1006–1082 (Fig. 4, B and C). Similar fractions of α1 photolabeling were recognized by anti-α1,1025–1040 (Fig. 4, B and D) whereas anti-α1,1011–1026 failed to immunoprecipitate significant amounts of peptide-associated labeling after tryptic digestion (as did control antibodies anti-α1,219–309 and anti-α1,339–354, Fig. 4D). Therefore cleavage occurred at the C-terminal end of epitope α1,1011–1026 at arginine 1022. According to our molecular mass estimate, the C-terminal cleavage site most likely corresponds to Lys-1083 (calculated molecular mass 7.2 kDa). Fig. 5 shows the approximate position of the smallest photolabeled tryptic fragments within the proposed folding structure of α1.

**DISCUSSION**

Benz(othi)azepine Labeling Occurs Near the DHP and PAA Interaction Sites of the α1 Subunit—We synthesized [3H]benziazem as a novel photoaffinity probe to selectively label the BTZ-binding domain with high yield. Specifically photolabeled proteolytic fragments derived from this domain contained transmembrane segments S6 of repeats III and IV together with extracellular and intracellular sequence stretches. Residues within these regions must therefore be in close contact with the bound drug molecule. A preliminary study (10) of photolabeling by azidobutyryl clentiazem, a photoaffinity probe with much lower affinity and labeling efficiency, also implicated the SS-S6 region of repeat IV in benzothiazepine binding, but the labeled peptides could not be specifically identified by SDS-PAGE and characterized because of the low level of specific labeling. Nevertheless, this earlier work adds further support for our conclusion that a component of the benz(othi)azepine receptor site is located in transmembrane segment S6 of domain IV.

Previous functional studies revealed that a membrane-permeable dibenziazem analogue can access its site only via an extracellular permeation pathway (24, 25). We therefore propose...
that the high affinity BTZ-binding domain is formed by residues within the photolabeled regions that can be accessed from the extracellular surface of the \( \alpha_{1} \) subunit.

Investigation of the molecular organization of the local anesthetic binding domain of voltage-gated Na\(^+\)-channels has shown that residues not directly involved in high affinity drug binding in segment IVS6 control drug access to the adjacent binding site (26). This indicates that drug access involves steric interactions with amino acids near the drug-binding domains. It is therefore possible that \([^3H]\)benziazem not only photoaffinity labels high affinity determinants for BTZ interaction but also adjacent residues modifying drug access to the binding domain.

As only one high affinity BTZ-binding domain exists on L-type Ca\(^{2+}\) channel \( \alpha_{1} \) subunits, the photolabeled regions must be located in close proximity to each other in the folded struc-
ture of α1. This closely resembles the topology of the DHP-binding domain, where high affinity determinants for drug interaction are also provided by repeats III and IV including the respective S6 segments (3, 4, 7, 9). Accordingly, the [3H]benziazem labeled fragments were also found to be photolabeled by different DHPs (3). In contrast, PAsAs only seem to bind to residues within IVS6 (6, 8). Considering that S6 segments are believed to contribute to the lining of the ion conducting pathway (6, 26), our data indicate that the binding domains for the three major classes of Ca²⁺ antagonists are located in close proximity to each other within pore-forming regions of L-type channel α1 subunits.

Antibody mapping of DHP photolabeled α1 subunit also revealed labeling within the connecting linker between segments S5 and S6 in repeat III resulting in a labeled 3-4-kDa tryptic peptide. Experiments with chimeric α1 subunits recently confirmed the importance of this region to confer DHP sensitivity (7). We found no evidence for [3H]benziazem labeling of this fragment that should be recognized by anti-α1\(\text{1011-1026}\). Similar to the bulky photoactive side chains of DHP photoligands (27), the relatively flexible (see legend to Fig. 1) photoactive benzophenone group may reach more peripheral residues of the BTZ-binding domain. Based on the absence of photolabeling in the I11S3–I11S6 linker in repeat III it therefore appears unlikely that this region contains major determinants for high affinity BTZ interaction.

Implications for Non-competitive Binding Interactions among Ca²⁺ Antagonist Drugs—Although identical tryptic fragments are photolabeled by DHPs and [3H]benziazem, the respective binding domains are not identical. In radioligand binding studies non-competitive interactions have been described between DHP and BTZ binding that require DHP and BTZ binding to the channel at the same time resulting in the formation of a ternary complex (1). Allosteric interactions have originally been proposed to account for these effects (1). However, the close association of all three binding domains within pore-forming regions of the channel may also allow steric interactions between these drugs. We have previously obtained evidence for an isosteric interaction between DHPs and BTZs in studies with the fluorescent Ca²⁺ antagonist DMBODIPY-BAZ (12, 28). The effects of the DHP (+)-isradipine on DMBODIPY-BAZ binding and fluorescence suggested that these drugs are located in close proximity to each other when simultaneously bound to the α1 subunit in a ternary complex. Our biochemical findings further support such a model.

Taken together we demonstrate that pore-forming regions of L-type Ca²⁺ channel α1 subunits in repeats III and IV provide a “hot spot” for the binding and action of chemically unrelated Ca²⁺ antagonists. Refining the structural organization of this region may not only help to provide insight into the molecular mechanism of Ca²⁺ antagonist action but may also aid in the development of organic channel blockers against other types of voltage-gated Ca²⁺ channels.