Azidobutyryl clentiazem, a new photoactivatable diltiazem analog, labels benzothiazepine binding sites in the α_1 subunit of the skeletal muscle calcium channel

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[³H]Azidobutyryl clentiazem, a new photoactivatable diltiazem derivative, has a higher binding affinity than azidobutyryl diltiazem. It can be covalently incorporated into the α_1 subunit of the skeletal muscle calcium channel by UV irradiation, which allows the benzothiazepine binding site to be determined. The photolabeled α_1 subunit and its proteolytic fragments were analyzed with a panel of sequence-directed antibodies. The results suggest that the linker region between segment S5 and S6 of domain IV is involved in benzothiazepine binding. This site is different from the dihydropyridine and verapamil binding sites.

[³H]Azidobutyryl clentiazem; Diltiazem-binding site; Photoaffinity labeling; Calcium channel; Sequence-directed antibody

1. INTRODUCTION

Selective calcium antagonists such as 1,4-dihydropyridines (e.g. nifedipine), phenylalkylamines (e.g. verapamil) and benzothiazepines (e.g. diltiazem) inhibit calcium influx through voltage dependent calcium channels. These drugs are widely used in the treatment of various cardiovascular disorders. Benzothiazepines, in particular, are quite useful because of a low side effect profile [1–3].

It is likely that the calcium antagonist drugs exert their effects by binding to distinct but allosterically coupled receptor sites of the voltage-dependent calcium channel [4]. These sites are part of the α_1 subunit of the calcium channel, as shown by photoaffinity labeling [5–10]. The regions participating in the formation of drug binding domains for phenylalkylamines and dihydropyridines have recently been identified within the primary structure of the skeletal muscle α_1 subunit after photoaffinity labeling using sequence-directed antibodies and defined proteolysis. The phenylalkylamine binding site is thought to be located at the intracellular surface of the membrane in the S6 segment of domain IV [11]. In contrast, the dihydropyridine binding site is located extracellularly, near the putative channel pore involving transmembrane helices S6 in domain III and IV [12,13].

It was shown in electrophysiological studies by using quarternary derivatives of diltiazem [14] and benzazepine [15], that the target of action of these compounds appears to be located on the extracellular portion of the α_1 subunit. However, the exact location of the benzothiazepine binding domain within the primary structure of the α_1 subunit remains to be elucidated. Photoaffinity labeling using an appropriate benzothiazepine derivative should be a promising avenue for this area. Earlier studies have shown that radiolabeled azidobutyryl diltiazem, a photoactivable benzothiazepine-type compound, covalently labels the α_1 subunit of the calcium channel [10].

In the present study, a part of the benzothiazepine binding site(s) in the voltage-dependent calcium channel has been localized within the α_1 subunit using a new

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Abbreviations: ABC, azidobutyryl clentiazem; EGTA, ethylenebis(oxyethylenenitrilo) tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperidine-*N'*-2-ethanesulfonic acid; RTT-membranes, rabbit transverse tubular membranes; CP, calcium channel peptide; WGA, wheatgerm agglutinin; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; Lys-C, lysyl endoprotease.

photoaffinity probe of a diltiazem analog. Here we describe an analysis of the photolabeled α_1 subunit before and after protease digestion by probing with a panel of sequence-directed antibodies. The results are compared to the known photolabeled sites for dihydropyridines and verapamil.

2. MATERIALS AND METHODS

2.1. Materials

[³H]Azidobutyryl clentiazem ([³H]ABC, 85 Ci/mmol), ABC and clentiazem were gifts from Tanabe Seiyaku Co., Osaka, Japan. [³H]Clentiazem (108 Ci/mmol) and diltiazem were gifts from Marion Merrell Dow Inc., Kansas City, MO. Lysyl endoprotease (Lys-C, *Achromobacter* protease I) was obtained from Wako Chemical Co., Osaka, Japan, and tosyl L-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin) (from bovine pancreas) and protein A-Sepharose 4B-CL were from Worthington and Pharmacia LKB, respectively.

2.2. Site-directed antibodies

Polyclonal antisera were raised in rabbits against synthetic calcium channel peptides corresponding to sequences of the skeletal muscle α_1 subunit (Fig. 1). Peptide CP-(1011 1026C), CP-(GC1381–1399), or CP-(1401–1415CG) were synthesized by the addition of a cysteine, glycylcysteine, or cysteinylglycine residue to the C- or N-terminus of CP-(1011–1026C), CP-(1381–1399), CP-(1401–1415), respectively. Peptide synthesis, coupling to porcine thyroglobulin, and antibody generation have been described [12].

2.3. Radioligand binding study

ABC was characterized by competition binding with [³H]clentiazem according to the method previously described [10]. In brief, rabbit skeletal muscle membranes (SM-membranes) enriched in L-type calcium channel antagonist receptors were incubated with 2 nM [³H]clentiazem for 60 min at 15°C in a buffer containing 50 mM Tris-HCl, pH 8.0 in the absence or presence of unlabeled ABC, clentiazem or diltiazem. Membrane-bound radioactivity was determined by a rapid filtration method. The data were analyzed with a nonlinear least squares LIGAND program originally described by Munson and Rodbard [16].

2.4. Photoaffinity labeling

Skeletal muscle membranes were homogenized and centrifuged at 100,000 × g for 30 min, the pellets were resuspended in the buffer (50 mM Tris-HCl, pH 8.4, containing 2 mM EGTA and four protease inhibitors: 0.25 mM phenylmethanesulfonyl fluoride, 1 mM iodoacetamide, 1 mM leupeptine and 1 μM pepstatin A) giving 10 mg/ml suspension. The membranes were incubated with 50 nM [³H]ABC in the absence or presence of unlabeled 0.1 mM diltiazem at 25°C for 60 min. The incubation mixture was then irradiated with UV light (Philips 38 W/TL lamp and 100 W/lamp) on ice at a distance of 10 cm for 20 min, and centrifuged at 100,000 × g for 40 min. The pellets were homogenized with the solubilization buffer (1% digitonin in 10 mM HEPES-Tris, pH 8.4, containing 185 mM KCl, 1.5 mM CaCl₂ and the four protease inhibitors), kept on ice for 60 min, and centrifuged at 100,000 × g for 45 min. The supernatant was irradiated again on ice with UV lights (Philips 38 W/TL lamp and 100 W/lamp) at a distance of 10 cm for 50 min. Labeled calcium channel proteins were purified using WGA-Sepharose 6MB [17], followed by size-exclusion chromatography with two columns of TSK G4000SW and TSK G3000SW, in the elution buffer (20 mM sodium phosphate, pH 7.0 containing 0.2% SDS) at a flow rate of 0.5 ml/min. Fractions containing the radiolabeled α_1 subunit were pooled, lyophilized and subjected to tryptic digestion and immunoprecipitation.

2.5. Protease digestion of purified [³H]azidobutyryl clentiazem-labeled α_1 subunit

For Lys-C digestion, the lyophilized [³H]ABC-labeled a1 subunit (90,000 dpm) was dissolved in water (2 ml) and the solution was dialyzed against 6M urea and subsequently against 0.003% Triton X-100 in 10 mM Tris-HCl (pH 7.5). The dialyzed sample was concentrated to 0.3 ml by lyophilization and the concentrate was digested with Lys-C (4 mg/ml) in 0.1 M Tris-HCl (pH 9.0) for 12 h at 37°C. For trypsin digestion, the lyophilized [³H]ABC-labeled α_1 subunit was dissolved in 0.4 M Tris-HCl, pH 8.0 and reduced with 1% 2-mercaptoethanol, followed by carboxymethylation with sodium iodoacetate as previously described [12]. The sample was dialyzed against 6 M urea and subsequently against 0.003% Triton X-100 in 10 mM Tris-HCl (pH 7.5). The dialyzed sample was concentrated to 0.3 ml by lyophilization and the concentrate was digested with trypsin (6.25 μg/ml) in 0.1 M Tris-HCl (pH 8.2) containing 2 mM CaCl₂ at 37°C. After 12 h, trypsin (6.25 μg/ml) was added and the digestion continued for 12 more hours. The digestion was terminated by the addition of diisopropyl phosphofluoridate to a final concentration of 5 mM.

2.6. Immunoprecipitation

Immunoprecipitation was carried out essentially as described [12]. Briefly, either digested or non-digested [³H]ABC-labeled α_1 was incubated with antibody-coupled protein A-Sepharose 4B-CL gel (100 μl) in RIA buffer (10 mM Tris-HCl, pH 7.2 containing 150 mM NaCl and 0.3% Triton X-100) at room temperature. After 2 h, the gel was washed with RIA buffer. Immunoprecipitated radioactivity was either directly determined by liquid scintillation counting after resuspension of the gel in 0.1 M sodium citrate (pH 3.0) or analyzed by SDS-PAGE after extraction with hot 1% SDS.

2.7. SDS-PAGE

The intact α_1 subunit was analyzed on SDS-PAGE using 7% polyacrylamide gels according to Laemmli [18]. For separation of the
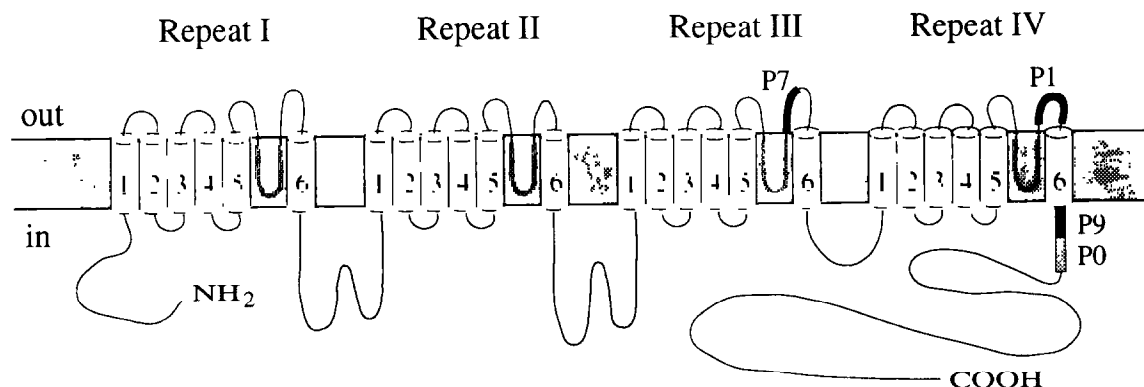

Fig. 1. Location of specific sequences on the α_1 of the rabbit skeletal muscle calcium channel. Antibodies were raised by synthetic peptides corresponding to the amino acid residues of 1011–1026 (P7), 1338–1351 (P1), 1381–1399 (P9) and 1401–1415 (P0).

digested fragments, the gel system described by Schaegger and von Jagow [19] was used. Detection of labeled peptides by gel slicing was carried out as described [12].

3. RESULTS AND DISCUSSION

In competition binding studies, ABC, like clentiazem and diltiazem, completely inhibited specific binding of [³H]clentiazem to the skeletal muscle membranes (Fig. 2). The equilibrium dissociation constants (K_d values) for ABC, clentiazem, and diltiazem were 60 ± 5, 44 ± 6, and 148 ± 12 nM, respectively, and the Hill slopes for these compounds were close to unity, viz., 0.99 ± 0.08, 1.01 ± 0.16, and 1.14 ± 0.2, respectively. These data indicate that ABC is a competitive inhibitor with a relatively high affinity for the diltiazem receptor site of the skeletal muscle calcium channel.

By photoirradiation, [³H]ABC was covalently incorporated into the 170 kDa polypeptide, the α_1 subunit of the calcium channel from rabbit skeletal muscle (Fig. 3). Specific incorporation was also demonstrated by another experiment in which the photo-incorporation was blocked in the presence of an excess amount of diltiazem, the prototypical and reversible benzothiazepine ligand for this receptor (Fig. 3). Efficient photolabeling was achieved by a two-step UV irradiation protocol which includes (1) irradiation of the membrane after 1 h incubation with [³H]ABC and (2) a second longer irradiation after removing free ligand and solubilizing with 1% digitonin.

The photolabeled samples were purified by affinity chromatography with WGA-Sepharose 6MB followed by size-exclusion chromatography to yield the [³H]ABC-labeled α_1 subunit as a major component. Partially purified labeled samples were digested with proteolytic enzymes (either Lys-C or trypsin). The cleavage mixture was then assayed with sequence-directed antibodies in order to identify those specific segments of α_1 where the radiolabeled ligand covalently binds. Fig. 4
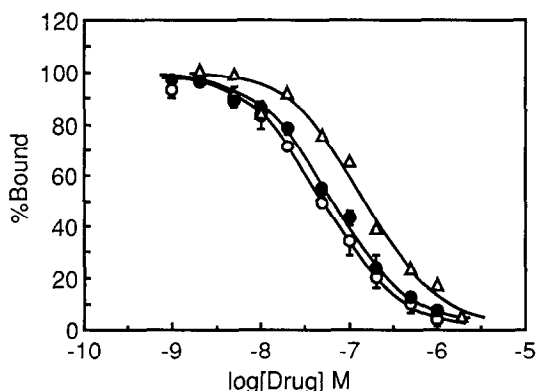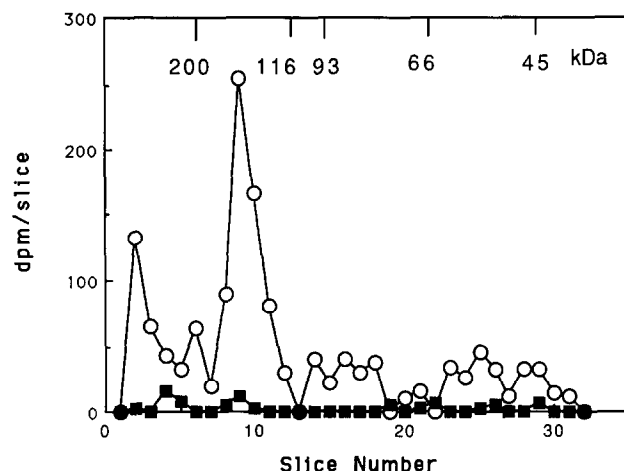
Fig. 3. Specific photoaffinity labeling of the diltiazem receptors with [³H]ABC. The SM-membranes were incubated with 50 nM [³H]ABC (open circles), or 50 nM [³H]ABC plus 0.1 mM diltiazem (filled squares). Calcium channel proteins were purified by WGA-Sepharose, treated with 50 mM NEM, and electrophoresed. The radioactivity was determined in 3 mm gel slices. M_r standards are shown.

shows the immunoprecipitation profile of non-digested (dotted bars), Lys-C digested (solid bars), and trypsin-digested (open bars) photoaffinity labeled α_1 subunit by several sequence-directed antibodies (see Fig. 1). The non-digested α_1 subunit was immunoprecipitated by all the antibodies. After digestion with Lys-C, anti-CP-(1338–1351) and anti-CP-(GC1381–1399). The antibodies were still able to bind radioactivity (15% and 18% of the specifically bound radioactivity associated with α_1, respectively). Contrary to the Lys-C digestion, none of the antibodies immunoprecipitated any labeled fragment after tryptic digestion. This is in clear contrast to previous observations that anti-CP-(1011–1026C) and anti-CP-(1338–1351) [12], or anti-CP-(1339–1354) and anti-CP-(1382–1400) [13] efficiently immunoprecipitated the tryptic fragments of the α_1 subunit that were labeled by photoactivable dihydropyridines such as diazipine [12], azidopine and PN200-110 [13], or by N-methyl-LU49888, a photoactivable verapamil [11], respectively. These results suggest that the region photolabeled by [³H]ABC is at a site distinct from dihydropyridines and verapamil.

Lys-C digestion of the photolabeled and purified sample generated several fragments of 25–50 kDa, 16 kDa, and 12 kDa as major fragments with minor fractions of 8 kDa, 4–5 kDa, and 2–3 kDa (Fig. 5). We found two major immunoprecipitated fragments of 12 kDa and 25 kDa by anti-CP-(1338–1351) antibody and anti-CP-(GC1381–1399) antibody but not by anti-CP-(1401–1415CG) antibody. However, after tryptic digestion no photolabeled fragment was detected by any of the three antibodies described above. It is predicted from the primary amino acid sequence of the α_1 subunit that Lys-C should give a 7.4 kDa polypeptide of amino acid (a.a.) 1337–1403 which includes the recognition
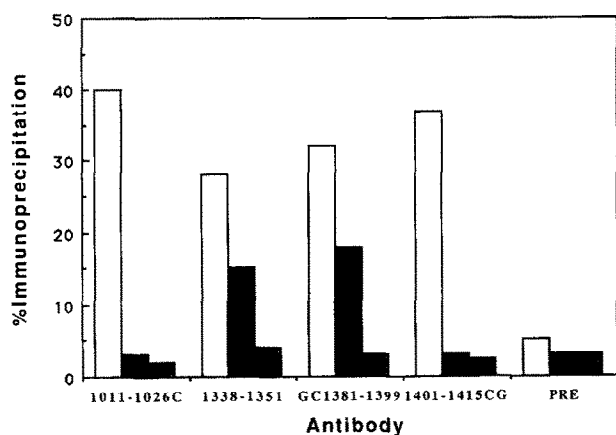
Fig. 2. Inhibition of specific [³H]clentiazem binding by diltiazem-type ligands. SM-membranes were incubated with 2 nM [³H]clentiazem in the presence of indicated concentrations of unlabeled clentiazem (○), ABC (●), or diltiazem (△).

Fig. 4. Immunoprecipitation of the [^{3}H]ABC-labeled α_1 subunit by various sequence-directed antibodies before and after protease digestion. Photolabeled α_1 subunit (open bars) and its digests with Lys-C (4 mg/ml; solid bars) or with trypsin (12.5 μg/ml; shaded bars) were immunoprecipitated with the antibodies against the CP indicated.

sites for the two antibodies described above. Thus the 12 kDa and 25 kDa fragments are probably the two partially digested fragments including the 7.4 kDa polypeptide. Together with the observation that the anti-CP-(1401–1415CG) antibody did not immunoprecipitate these fragments, the fragments containing the 7.4 kDa polypeptide and the additional residues extend towards the N-terminal end of α_1 subunit. The corresponding amino acids are considered as a.a. 1292–1403 and a.a. 1173–1403 for 12 kDa and 25 kDa fragment, respectively.

No antibodies immunoprecipitated any labeled fragments after tryptic digestion which can generate 6.2 kDa (a.a. 1337–1389) or 7.4 kDa (a.a. 1337–1403), as demonstrated previously [12], from the fragments of 12 kDa and 25 kDa. Therefore, the photolabeled site with
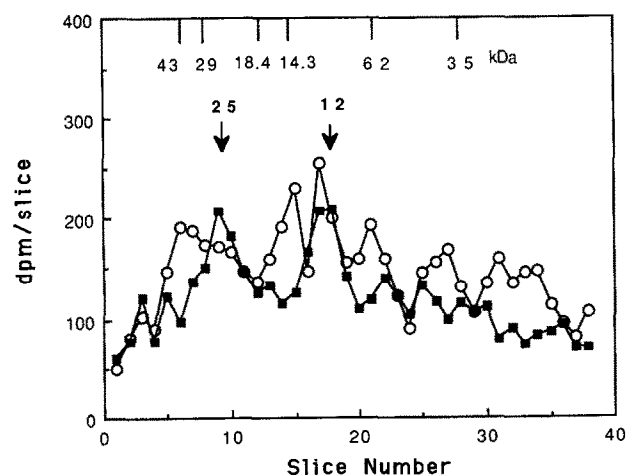

Fig. 5. Analysis of immunoprecipitated [^{3}H]ABC-labeled fragments from Lys-C digestion by SDS-PAGE. ABC-labeled α_1 was digested with Lys-C (4 μg/ml). Aliquots of the digest were separated by SDS-PAGE (open circles) or after immunoprecipitation with anti-CP-(GC1381–1399) (filled squares) in the gel system described by Schaegger and von Jagow [19].

[^{3}H]ABC is located between a.a. 1292 and 1403 excluding a.a. 1337–1403. It is likely that the 25 kDa fragment is a precursor of the 12 kDa fragment and both of the fragments have the same labeled site with [^{3}H]ABC, but further work is necessary for proof.

This is the first demonstration by photoaffinity labeling that the binding site for the diltiazem-type calcium antagonist is in domain IV, but at a site different from dihydropyridines and phenylalkylamines [11–13]. Additional studies are required in order to identify the exact binding sites for diltiazem-type antagonists. ABC has proven to be a promising candidate for probing specific regions.

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