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Identification of 1,4-dihydropyridine binding domains within the primary structure of the α_1 subunit of the skeletal muscle L-type calcium channel

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Calcium channel blockers are drugs that bind to the α_1 subunit of L-type calcium channels and selectively inhibit ion movements through these channels. Determination of the mechanism of channel blockade requires localization of drug-binding sites within the primary structure of the receptor. In this study the 1,4-dihydropyridine-binding site of the membrane bound receptor has been identified. The covalently labeled receptor was purified and digested with trypsin. The labeled peptide fragments were immunoprecipitated with sequence-directed antibodies. The data indicate the existence of at least three distinct dihydropyridine-binding domains within the primary structure of the α_1 subunit.

Calcium channel blocker; 1,4-Dihydropyridine receptor

1. INTRODUCTION

L-Type calcium channels are complex oligomeric membrane proteins that regulate voltage-dependent influx of calcium into excitable cells [1,2], and are one of the key components of the excitation contraction coupling machinery [3]. These channels are also the receptors for the calcium antagonists or calcium channel blockers [4]. These drugs play an important role in the treatment of many different cardiovascular disorders, particularly hypertension and angina pectoris [5]. All calcium channel blockers, in spite of their different chemical structures, specifically bind to the α_1 subunit of the L-type calcium channels [6–8] and prevent calcium influx into the cells. The 1,4-dihydropyridines (DHPs) are one of the chemical groups of calcium channel blockers [5].

Electrophysiological studies have indicated that DHPs act on calcium channels only when they are applied to the extracellular compartment [9,10]. These data suggest that the DHP-binding site(s) is either located at the extracellular portion of the α subunit or is accessible only from the outside of the cell.

In the first major attempt to identify the DHP-binding site, the strategy involved covalent labeling of the solubilized and partially purified α_1 subunit with either [^{3}H]azidopine or [^{3}H]nitrendipine, digestion of the receptor with proteases, purification and sequencing of the labeled peptide fragments [11]. The amino acid sequences of the purified and labeled peptides corresponded to amino acid sequences located between amino acid residues 1,390–1,437; 13–18 and 712–722 in the deduced skeletal muscle L-type calcium channel α_1 subunit sequence [12]. It was suggested that the DHP-binding site was located distal to the S6 transmembrane segment of repeat IV at the cytosolic region of the α_1 subunit, close to a putative cytosolic calcium-binding site (EF hand) and at intracellular segments of repeat I, close to the cytosolic amino-terminus [11]. In contrast to this, and in agreement with recent electrophysiological studies [9,10], immunoprecipitation of labeled receptor fragments with sequence-directed antibodies resulted in a different conclusion [13–15]. After labeling of partially purified receptors with [^{3}H]diazipine, [^{3}H]azidopine or [^{3}H]PN200–110, two putative extracellular peptide fragments located adjacent to transmembrane segments S6 of repeats III and IV were identified. It was suggested that both of these drug-binding domains are involved in the DHP-binding site of L-type

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Abbreviations: IAA, iodoacetamide; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIA, radioimmunoassay.

calcium channels [15]. While the methods of identification of covalently labeled peptide fragments were different, all previous studies utilized solubilized and partially purified receptor protein, which has approximately an order of magnitude lower affinity for DHPs than membrane-bound receptors [6].

In this study the receptors were photoaffinity labeled in their native, membrane-bound and high-affinity form. After these receptors were purified and digested with trypsin, three distinct covalently labeled DHP-binding domains were identified with sequence-directed antibodies.

2. MATERIALS AND METHODS

2.1. *Preparation of skeletal muscle membranes*

Membranes enriched in transverse tubules were isolated from fresh rabbit skeletal muscle according to Glossmann and Ferry [16]. The density of DHP-binding sites in membranes was determined by radioligand-binding assay as previously described [4]. These membranes had a maximum binding capacity (B_{max}) of 5–15 pmol/mg protein and a K_D of 0.3–0.5 nM for (+)[^{3}H]PN200–110.

2.2. *Photoaffinity labeling and solubilization of the membrane-bound receptors*

Membranes (600 mg) were diluted to a protein concentration of 10 mg/ml with a labeling buffer (50 mM Tris-HCl, pH 7.4, at 24°C containing the following protease inhibitors: 0.25 mM PMSF, 1 mM IAA, 1 μM leupeptin and 1 μM pepstatin). The membrane suspension was homogenized in a Thomas C size homogenizer and centrifuged at 100,000 × g for 40 min. The pellet was resuspended in the labeling buffer to obtain a 10 mg/ml protein concentration. This suspension was incubated at 24°C with 2 nM [^{3}H]azidopine (specific activity 50 Ci/mmol, Amersham Int.). Non-specific labeling was measured in a sample that also contained 2 μM unlabeled racemic PN200–110. At the end of the incubation, the samples were placed on ice and irradiated with a Philips 38W/TL UV lamp from a distance of 10 cm for 15 min. The irradiated samples were centrifuged at 100,000 × g for 40 min. The pellet was diluted with a solubilization buffer that contained 1% digitonin, 10 mM HEPES-Tris, pH 7.4, at 4°C, 185 mM KCl, 1.5 mM $CaCl_2$, 0.25 mM PMSF, 1 mM IAA, 1 μM leupeptin and 1 μM pepstatin and incubated on ice for 60 min. The insoluble materials were removed by centrifugation at 100,000 × g for 50 min. The supernatant-containing solubilized membrane proteins was used in subsequent studies.

2.3. *Wheat germ lectin affinity chromatography*

The solubilized proteins were incubated for 3 h at 4°C with 25 ml of WGL-Sepharose 6MB (Pharmacia Fine Chemicals, Uppsala, Sweden) that had previously been equilibrated with the solubilization buffer. The WGL-Sepharose 6MB was packed into a 1.5 cm × 30 cm column, washed with 50 ml solubilization buffer and then with 700 ml of solubilization buffer containing a reduced concentration (0.1%) of digitonin. *N*-Acetylglucosamine (400 mM) dissolved in this buffer was used to elute glycoproteins from the column. Fractions were collected and the elution of labeled proteins was monitored by measuring the radioactivity in 50 μl aliquots taken from each fraction. The fractions containing the highest radioactivity were combined, lyophilized and retained for subsequent experiments.

2.4. *Purification of the [^{3}H]azidopine-labeled α_1 subunit*

Partially purified and lyophilized proteins were dissolved in distilled water and reduced with 1% 2-mercaptoethanol for 15 min at 24°C before carboxymethylation with iodoacetamide for 1 h at 24°C. The photoaffinity-labeled α_1 subunit was then further purified by size exclusion chromatography on tandemly connected TSKgel G columns (Tosoh Corp., Japan; TSKgel SW guard column, 75 × 7.5 mm ID, TSKgel G3000SW column, 600 × 7.5 mm ID; and TSKgel G4000SW column, 600 × 7.5 mm ID) in 20 mM sodium phosphate buffer, pH 7, containing 0.2% sodium dodecylsulfate at a flow rate of 0.5 ml/min. 1 ml fractions were collected and the radioactivity was measured in 50 μl aliquots taken from each fraction. The peak fractions were combined, lyophilized and stored at −70°C. Samples of the combined fractions were subjected to SDS-PAGE using 5 - 15% polyacrylamide gradient gels. The gels were stained with Coomassie blue. The radioactivity was determined in gel slices according to the method of Sunahara et al. [17].

2.5. *Generation of proteolytic fragments of purified [^{3}H]azidopine-labeled α_1 subunit*

The purified [^{3}H]azidopine-labeled α_1 subunit preparation containing approximately 8×10^5 dpm was dissolved in distilled water by ultrasonication. The soluble protein was first dialyzed against 6 M urea and then against distilled water before lyophilization. The concentrated sample was digested with trypsin (50 μg/ml) for 12 h at 37°C in 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$. The reaction was terminated by the addition of diisopropyl phosphofluoridate to a final concentration of 5 mM [13].

2.6. *Immunoprecipitation of covalently labeled receptor fragments by sequence-directed antibodies*

Sequence-directed polyclonal antibodies were generated as previously described [13]. Antibodies P0, P1, P7 and P10 were raised against synthetic peptides corresponding to amino acid residues 1,401–1,414

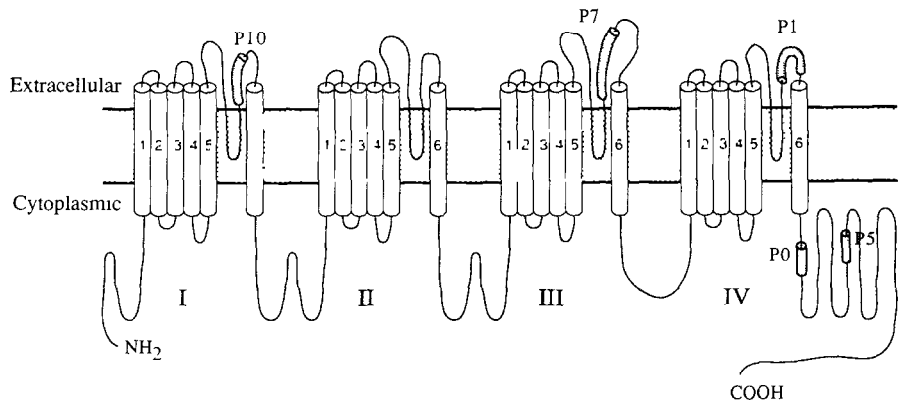

Fig. 1. Location of amino acid sequences in a membrane-folding model of the skeletal muscle L-type calcium channel α_1 subunit which were selected for generation of sequence-directed antibodies P0, P1, P5, P7 and P10.

(P0), 1,338–1,351 (P1), 1,011–1,026 (P7) and 264–280 (P10) in the deduced amino acid sequence of the skeletal muscle α_1 subunit [12]. Antibody P5 was raised against a synthetic peptide corresponding to amino acid residues 1,691–1,701 of the cardiac L-type calcium channel α_1 subunit [18]. This sequence is highly homologous to amino acid residues 1,566–1,582 in the skeletal muscle α_1 subunit [12]. The location of amino acid sequences within the primary structure of the skeletal muscle L-type calcium channel α_1 subunit is illustrated in Fig. 1.

The antibodies were bound to protein A–Sepharose 4B by incubating 1 vol. of serum with 1 vol. of swollen gel in RIA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% Triton X-100) for 2 h at 4°C. After washing the gel with ice-cold RIA buffer, tryptic fragments of the receptor were added. The peptide fragments were incubated with the antibodies for 2 hours at room temperature and the gel was washed with RIA buffer. Immunoprecipitated radioactivity was determined by SDS-PAGE after denaturation of the resin in SDS sample buffer and counting radioactivity in gel slices.

3. RESULTS

3.1. *Photoaffinity labeling of the membrane-bound receptors with [^{3}H]azidopine*

Skeletal muscle membranes enriched in DHP receptors were incubated with [^{3}H]azidopine at a receptor-to-ligand ratio of 50:1. After equilibrium binding was achieved (60 min), photoincorporation of [^{3}H]azidopine was induced by UV irradiation. The free ligand, except the one entrapped in the pellet, was removed by centrifugation. The free ligand in the pellet was diluted and gradually removed by solubilization and the subsequent chromatographic steps.

3.2. *Purification of the labeled α_1 subunit*

The photoaffinity-labeled α_1 subunit was first purified by wheat germ lectin affinity chromatography. Incorporation of [^{3}H]azidopine into the partially purified α_1 subunit was verified by SDS-PAGE, gel slicing and by counting radioactivity in the gel slices (data not shown). These partially purified receptors were carboxymethylated and solubilized with SDS before final purification by high performance liquid chromatography using size exclusion columns. The size exclusion chromatography of the solubilized and denatured receptors resulted in three radioactive peaks. Each peak appeared to represent specific labeling (Fig. 2, upper panel). To determine which peak contained the α_1 subunit, the peak radioactive fractions from the totally labeled preparation were pooled and a sample was subjected to SDS-PAGE. The radioactivity was counted in gel slices. The second and third radioactive peaks that eluted at 27–30 ml and 37–39 ml from the size exclusion columns did not contain intact DHP receptors or other proteins detectable with Coomassie blue staining. In these cases the radioactivity migrated faster than the dye and eluted from the gel (Figure 2, lower panel). On the other hand, the first radioactive peak, that was eluted at 20–23 ml from the size exclusion columns, did contain the photoaffinity-labeled, 170 kDa α_1 subunit (Fig. 2, lower panel). These fractions were used either directly or after tryptic digestion for immunochemical analysis.

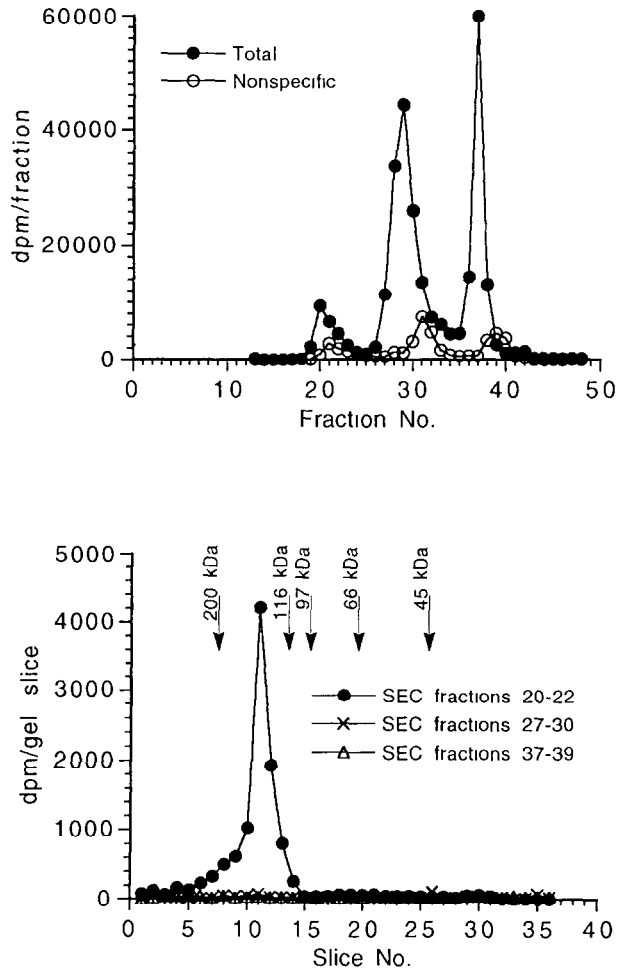

Fig. 2. Identification of intact, [^{3}H]azidopine-labeled α_1 subunits in purified receptor preparations. The radioactivity present in the fractions collected after injection of totally and non-specifically labeled receptors into size exclusion columns is shown in the upper panel. Size exclusion chromatography (SEC) fractions 20–22, 27–30 and 37–39 were pooled and aliquots containing 10,000 dpm were subjected to SDS-PAGE. The radioactivity present in 3 mm gel slices is illustrated in the lower panel.

3.3. *Identification of photoaffinity-labeled peptide fragments with sequence-directed antibodies*

The purified photoaffinity-labeled α_1 subunit preparation was immunoprecipitated with all sequence-directed antibodies used in this study (P0, P1, P5, P7 and P10). In contrast, the photoaffinity-labeled tryptic fragments of the α_1 subunit were immunoprecipitated by only three of these antibodies, P1, P7 and P10 (Fig. 3). Approximately 60% of specific [^{3}H]azidopine labeling was immunoprecipitated with these three antibodies. There was no significant amount of radioactivity immunoprecipitated with antibodies P0 and P5 or with preimmune serum.

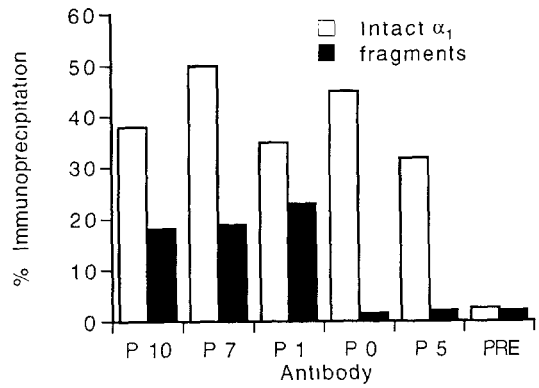

Fig. 3. Immunoprecipitation of [^{3}H]azidopine-labeled α_1 subunit by sequence-directed antibodies (P0, P1, P5, P7 and P10) before (open bars) and after digestion with trypsin (filled bars) PRE, preimmune serum.

SDS-PAGE analysis of the immunoprecipitated, labeled peptides revealed that anti-P7 and anti-P1 antibodies recognized a 3.5 kDa and a 6.2–7.4 kDa fragment, respectively, as has previously been shown after labeling of the DHP receptor with [^{3}H]diazipine [13] or [^{3}H]azidopine [14]. In addition, anti-P10 antibody recognized a 10 kDa photoaffinity-labeled fragment. The location of potential tryptic cleavage sites near the P10 sequence suggests that the 10 kDa-labeled tryptic fragment resulted from cleavage at Arg-262 and Lys-339 of the α_1 subunit, and represents the transmembrane segment S6 of domain I and the adjacent extracellular peptide loop.

4. DISCUSSION

Identification of drug-binding sites within the primary structure of the α_1 subunit of the L-type calcium channels is necessary for determination of the molecular mechanism of action of calcium channel blockers. A considerable effort has been made to achieve this goal [11,13,14,19].

In the present study, the 1,4-dihydropyridine-binding domains of the skeletal muscle L-type calcium channels were determined after photoaffinity labeling of membrane-bound receptors. [^{3}H]Azidopine has approximately 10-times higher affinity (K_D = 0.3 nM) to membrane-bound receptors than to partially purified receptors (K_D = 3 nM) [6]. Therefore, the membrane-bound receptors required 10-times lower concentration of the labeled ligand than the partially purified receptor preparations for the same fractional occupancy. The possibility of non-specific labeling was reduced by maintaining a high (50:1) ratio between receptor and the labeled ligand concentrations. Under such circumstances, the non-specific labeling was less than 10% of the total labeling. Furthermore, the non-specifically labeled membrane components were separated from the labeled receptors during subsequent solubilization and purification steps.

Identification of labeled peptides by immunoprecipitation with sequence-directed antibodies has proven to be a powerful procedure in determination of drug receptor sites [13,14,19]. We have successfully used this procedure and identified three distinct [^{3}H]azidopine-labeled polypeptides. These are located on peptide loops between transmembrane segments S5 and S6 of repeats I, III and IV. All three domains are suggested to be at the extracellular opening of the channel pore based on a transmembrane-folding model of the α_1 subunit [15]. Two of these DHP 1,4-dihydropyridine-binding domains have been identified before [13,14] but the third one, which is located on repeat I, has not. Therefore, our results confirm previous findings suggesting that

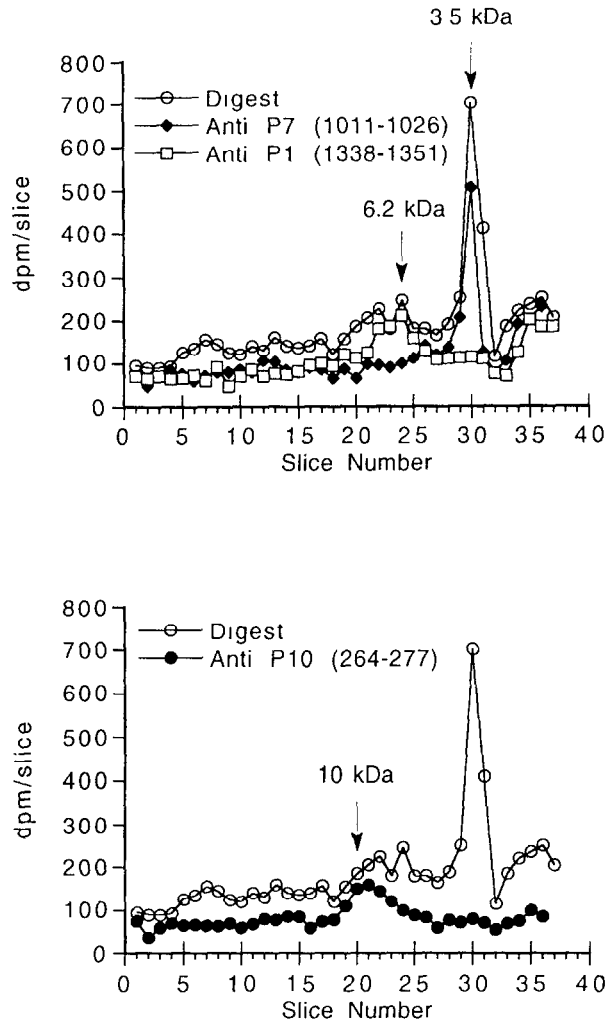

Fig. 4. Identification of [^{3}H]azidopine-labeled tryptic fragments of the α_1 subunit. Proteolytic fragments were separated by SDS-PAGE and the radioactivity (dpm) was determined in 3 mm gel slices. The radiolabeled peptides, (10 kDa, 6.2 kDa and 3.5 kDa) present in the digest before (○) and after immunoprecipitation with sequence-directed antibodies anti-P7 (◆), anti-P1 (□) and anti-P10 (●) are indicated by arrows.

putative extracellular segments of the α_1 subunit located between transmembrane segments S5 and S6 of repeats III and IV, that may fold into the pore of the channel, represent-binding domains of the 1,4-dihydropyridine receptor [15]. However, our data refine the localization by identifying a third putative extracellular segment of the α_1 subunit located between transmembrane segments S5 and S6 of repeat I, which we feel participates in the formation of the DHP receptor. This 'peptide loop' may fold into the channel pore together with the other DHP-binding loops. Thus, we suggest that the DHP receptor consists of at least three distinct binding domains located distal from each other in the primary structure of the receptor but may be close to each other in the three-dimensional structure of the α_1 subunit. This is not unexpected since the existence of multi-subsite receptors has been postulated for several drugs and hormones [20].

Our data suggest that the DHP receptor consists of at least three distinct drug-binding domains, and that 1,4-dihydropyridines are multivalent ligands that bind to more than one receptor subsite simultaneously and may act as reversible cross-linkers of peptide loops [21]

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