Photochemical localization of the semotiadil binding region within the cardiac Ca^{2+} channel α1 subunit. Comparison with the skeletal muscle counterpart

Nariaki Ii[a], Akihiko Kuniyasu[a], Kohichi Kawahara[a], Toshiro Shibano[b], Arnold Schwartz[c], Hitoshi Nakayama[a,*]

[a] *Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Ohe Honmachi, Kumamoto 082-0973, Japan*
[b] *New Product Research Laboratories II, Daiichi Pharmaceutical Co. Ltd., 1-16-13, Kita-Kasai, Edogawa-Ku, Tokyo 134-0081, Japan*
[c] *Insitiute for Molecular Pharmacology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0828, USA*

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Abstract We have previously identified the binding region of a new Ca^{2+} antagonist semotiadil in the skeletal muscle Ca^{2+} channel. To the same semotiadil derivatives, the cardiac counterpart showed distinct and different binding characteristics: semotiadil and its photoaffinity analog D51-4700 inhibited [^{3}H]PN200-110 binding to cardiac membrane preparations with IC_{50} values of 13–20 μM, which are 10 times higher than those in skeletal muscle. Hill slopes of the binding inhibition were 0.94–1.0 for the cardiac channels compared to 0.63–0.67 for the skeletal muscle channels. A possible explanation for the difference is that the semotiadil binding site is differently conferred in cardiac and skeletal muscle Ca^{2+} channels. To reveal this within the primary structure, photoaffinity labeling of cardiac membranes was employed. [^{3}H]D51-4700 was photoincorporated in several polypeptides but only the α1 subunit of the Ca^{2+} channel was photolabeled in a specific manner. Antibody mapping of the [^{3}H]D51-4700-labeled α1 subunit with several anti-peptide antibodies revealed that the labeled site was located solely in a peptide fragment between Cys^{1461} and Lys^{1529}. This region encompasses the labeled site of skeletal muscle, but contains several non-identical amino acid residues, which may participate in expressing different binding characteristics between the two muscle type Ca^{2+} channels.

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Key words: Semotiadil; Binding site; Calcium channel; Cardiac muscle; Photoaffinity labeling

1. Introduction

Ca^{2+} antagonists bind to L-type Ca^{2+} channels and block the entry of extracellular Ca^{2+}. Three classes of Ca^{2+} antagonists, 1,4-dihydropyridines (DHP), phenylalkylamines (PAA) and benzothiazepines (BTZ), bind to different regions on the α1 subunit of Ca^{2+} channels [1,2] as shown by photoaffinity labeling and molecular biological studies. As to the DHP binding site, not only transmembrane segment IVS6 but also IIIS6 and IIIS5 have been identified as molecular determinants of binding [3–8]. This is consistent with photolabeling results [9,10]. With regard to the PAA site, photolabeling studies identified IVS6 as the binding site [11]. Molecular mutagenesis studies showed that IIIS6 is a molecular determinant of high affinity binding [12–14]. With regard to the BTZ site, IVS6 [15] and the connecting loop between IVS5 and IVS6 [16] are constituents of the binding site. Taken together, IVS6 must represent a common and important region, although not sufficient, for the binding site of the three classes of Ca^{2+} antagonists. These results can also explain the well-known allosteric interactions with one another [17].

Semotiadil, a new Ca^{2+} antagonist with the 1,4-benzothiazine ring structure, shows different pharmacological actions than the conventional drugs. For example, semotiadil is longer-lasting than diltiazem and nifedipine [18,19]. In addition, semotiadil shows a higher selectivity for blood vessels compared with cardiac tissues than diltiazem but a lower selectivity than nifedipine [18,19]. Semotiadil has a negative allosteric interaction with DHP, PAA and BTZ in skeletal muscle Ca^{2+} channels [20–22] but equivalent data are not available for the cardiac Ca^{2+} channels. Therefore, localization of the semotiadil binding site is an interesting and potentially important subject. Initially we reported photoaffinity labeling of skeletal muscle Ca^{2+} channels using [^{3}H]D51-4700, a photoaffinity analog of semotiadil. This reagent labeled a region that overlapped with but was not identical to those for the conventional drugs [23]. When semotiadil and D51-4700 were applied to the cardiac Ca^{2+} channels, their binding characteristics were distinct from the skeletal muscle counterpart, suggesting that the semotiadil binding site is conferred differently between the two L-type channels. In order to reveal this within the primary structure, we employed photoaffinity labeling of the cardiac Ca^{2+} channels with [^{3}H]D51-4700. In this paper, we describe the localization of the photolabeled site of the cardiac Ca^{2+} channels and compare the semotiadil binding site for the cardiac Ca^{2+} channels with the skeletal muscle counterpart.

2. Materials and methods

2.1. Materials

[^{3}H]D51-4700 (77.6 Ci/mmol) was synthesized as described [24]. Semotiadil was obtained from Daiichi Pharmaceutical Co., Ltd. Enzymes and chemicals were obtained from the following sources: *Achromobacter lyticus* protease I (Lys-C) and digitonin from Wako Pure Chemicals (Osaka, Japan); protein A-Sepharose CL-4B and WGA-Sepharose 4B from Pharmacia Biotech Inc.; [^{3}H](+)PN200-110 (83.0 Ci/mmol) from NEN; prestained molecular weight standard-low from Life Technologies, Inc.; unstained high molecular weight standard from Bio-Rad; scintillation cocktail ACS II from Amersham; dimethyl pimelimidate from Pierce.

2.2. Anti-peptide antibodies

Polyclonal antibodies, raised against synthetic peptides corresponding to particular regions of rabbit skeletal muscle α1 subunit sequence [25], were used. We confirmed that the corresponding amino acid

*Corresponding author. Fax: (81) (96) 372-7182.
E-mail: jin@gpo.kumamoto-u.ac.jp

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sequences were almost identical to those of porcine cardiac muscles by RT-PCR experiments. The peptides of the skeletal muscle channels we used and their corresponding sequence to the cardiac channels are as follows: P9, skeletal 1382–1400 for cardiac 1507–1525 with no sequence difference; P0, skeletal 1401–1414 for cardiac 1526–1539 with one substitution of A for R at the 1404 position; P7, skeletal 1011–1026 for cardiac 1142–1157 with two substitutions of Q for E and A for S at the 1018 and 1023 positions, respectively. Sequences of the three cardiac peptides from porcine hearts were totally identical to those [26] from rabbit hearts. Methods for raising antibodies have been described previously [23,9].

2.3. Preparation of immunoaffinity beads of monoclonal antibody

Monoclonal antibody was purified from ascites by the usual method using protein A-Sepharose. The ascites were produced by injection of hybridoma 7H7 which was a gift from Dr. M. Takahashi (Mitsubishi Kasei Institute of Life Sciences). The hybridoma 7H7 produces monoclonal antibody against the fusion protein containing a 549 amino acid coding region of the C-terminus in the α1 subunit of the cardiac Ca^{2+} channel; the preparation of the fusion protein has been described in the literature [27]. Affi-Gel 10 was used to immobilize the purified IgG.

2.4. Binding assays

The inhibition assay of semotiadil and D51-4700 to the [^{3}H](+)PN200-110 binding to the Ca^{2+} channel preparations was performed essentially as described by Nakayama et al. [21] with some modifications. Briefly, sarcolemmal-rich membranes from porcine hearts (40 μg protein, 0.04 pmol of [^{3}H](+)PN200-110 binding sites; 0.04 nM) were incubated with 0.3 nM of [^{3}H](+)PN200-110 in the dark for 40 min at 37°C in assay buffer (50 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$, 0.1 mM PMSF) in the presence or absence of varying concentrations of semotiadil and D51-4700. For the assay of triad membranes (5 μg protein, 0.1 pmol of [^{3}H](+)PN200-110 binding sites; 0.1 nM) which was prepared from rabbit skeletal muscle as described by Mitchell et al. [32], 2 nM of [^{3}H](+)PN200-110 was used. In order to obtain accurate IC_{50} values, the [^{3}H](+)PN200-110 concentrations used above were around K_d values for the two Ca^{2+} channels and were kept in excess over the receptor concentrations. Membrane-bound radioactivity was determined by a rapid filtration. All assay were performed in triplicate. The data were analyzed with a non-linear least squares LIGAND program originally described by Munson and Rodbard [33].

2.5. Photoaffinity labeling and purification of α1 subunit of porcine cardiac Ca^{2+} channel

Sarcolemmal-rich membranes were isolated from porcine hearts as described previously [28]. The membranes (80–100 pmol of [^{3}H](+)-PN200-110 binding sites, 80 mg of proteins) were incubated with 200 nM [^{3}H]D51-4700 in 20 ml of binding buffer (25 mM Tris-HCl (pH 7.4), 0.1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 0.5 mM iodoacetamide) in the presence or absence of 10 μM semotiadil at 30°C for 60 min. The incubation mixture was transferred into a glass Petri dish on ice, and irradiated for 20 min with a 100 W black light/blue lamp (Ultra-Violet Products, San Gabriel, CA) at a distance of 10 cm. After photolysis, [^{3}H]D51-4700-labeled Ca^{2+} channels were solubilized in 1% (w/v) digitonin and purified by affinity chromatography on WGA-Sepharose 4B according to the described method [28]. After solubilization, all the subsequent procedures were performed at 4°C and all the buffer contained five protease inhibitors (0.1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 0.5 mM iodoacetamide). The eluate from WGA-Sepharose 4B was then purified by immunoaffinity chromatography. This step was essential to isolate the Ca^{2+} channel. The WGA-Sepharose eluate was loaded on a 7H7-agarose. The gel was washed with buffer (20 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, 0.2M NaCl, 0.1% digitonin) and eluted with elution buffer (0.1 M glycine-HCl (pH 3.0), 0.2 M NaCl, 0.1% digitonin). The eluate was immediately adjusted to pH 7.4 by addition of 1 M Tris-HCl (pH 9.0). Peak fractions were pooled and dialyzed against 2 mM Tris-HCl (pH 7.4) followed by lyophilization. The photolabeled and purified protein was then subjected to carboxymethylation and photolabeled α1 subunits were further purified by gel permeation liquid chromatography as described [23]. Fractions corresponding to the α1 subunit were pooled, lyophilized and stored at −30°C until use.

2.6. Proteolytic of [^{3}H]D51-4700-labeled α1 subunit

The photolabeled α1 subunit was dissolved in deionized water (0.5 ml) and dialyzed against 6 M urea as described [9], followed by dialysis against 0.01% Triton X-100 for 6 h. The sample was digested with Lys-C (100 μg/ml) in 50 mM Tris-HCl (pH 9.0) containing 0.05% (w/v) SDS and 0.01% (v/v) Triton X-100 (final volume of 100 μl) at 37°C for 6 h.

2.7. Immunoprecipitation

Antibodies were bound to protein A-Sepharose CL-4B gel by incubating 1 volume of antiserum with 1 volume of the swollen gel in the buffer A (10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.1% (v/v) Triton X-100 and 1 mg/ml bovine serum albumin) for 2 h at 4°C. The gel was washed with ice-cold buffer A before addition of digested or non-digested [^{3}H]D51-4700-labeled α1 subunits. After incubation for 2 h at room temperature, the gel was washed with buffer A. Immunoprecipitated radioactivity was directly determined by liquid scintillation counting of the protein A-Sepharose CL-4B gel with 100 mM sodium citrate (pH 3.0). Immunoprecipitated labeled fragments were extracted from the gel with a sampling buffer for SDS-PAGE (50 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 12% (v/v) glycerol) for 3 min at 90°C and analyzed by SDS-PAGE.

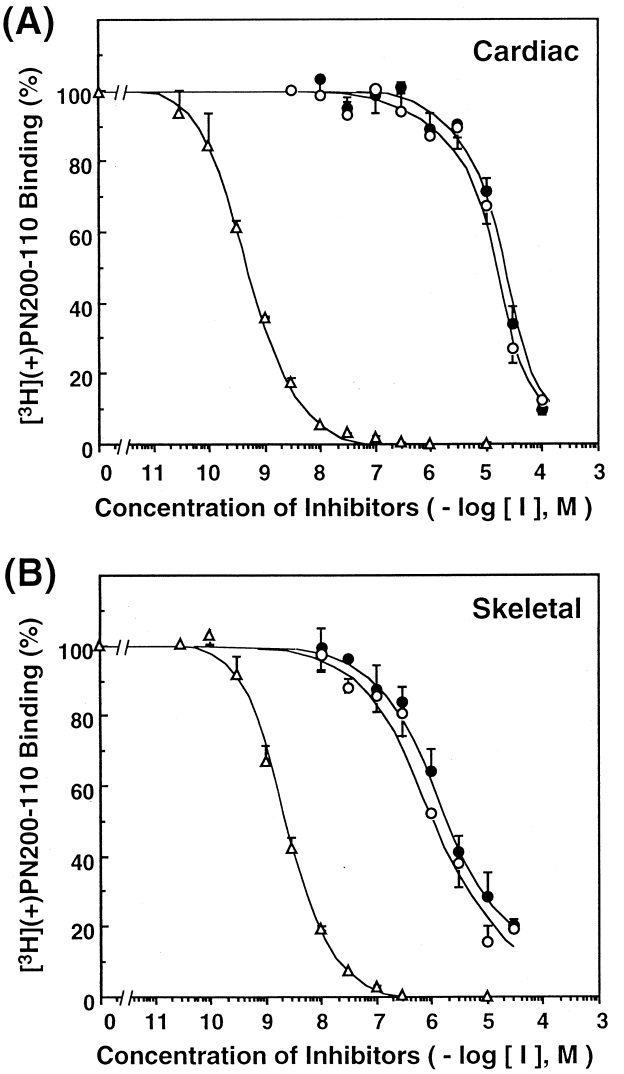

Fig. 1. Inhibition of [^{3}H](+)PN200-110 binding to the Ca^{2+} channel preparations by semotiadil and its photoaffinity analog. Porcine cardiac sarcolemmal-rich membranes (A) and rabbit skeletal triad membranes (B) were used. Semotiadil (○) and D51-4700 (●) are the tested drugs and unlabeled (±)PN200-110 (△) is used as control.

To determine the size of immunoprecipitated fragments, the antibody-protein A Sepharose complex was crosslinked with dimethyl pimelimidate as described by Schneider et al. [29].

2.8. SDS-PAGE and radioluminography

Intact α1 subunits were analyzed on SDS-PAGE using a 8% polyacrylamide gel according to Laemmli [30] and a sampling buffer (10 mM Tris-HCl (pH 7.6), 1% (w/v) SDS, 20 mM dithiothreitol, 4 mM ethylenediaminetetraacetic acid and 2% (w/v) sucrose). For separation of proteolytic fragments, the gel system described by Schägger and von Jagow [31] (4% stacking gel, 10% spacer gel and 16.5% separating gel, 3 or 6% cross-linking) was used. For visualization of the tritiated proteins and peptides, a more sensitive method ('radioluminography') than fluorography was used as previously described [23].

3. Results

3.1. Binding

Fig. 1 shows the effects of semotiadil and its photoaffinity analog D51-4700 on the binding of [^{3}H](+)PN200-110 to the porcine cardiac and rabbit skeletal muscle membrane preparations. Both compounds inhibited the [^{3}H](+)PN200-110 binding but dose dependences were different between the cardiac and skeletal muscle preparations. IC_{50} values of semotiadil and D51-4700 to the cardiac muscle membranes were 20 μM and 13 μM, respectively, while those to the skeletal muscle membranes were 2.0 and 1.5 μM. The data show that the inhibitions of semotiadil analogs to the cardiac membranes are approximately 10 times less effective than those to the skeletal muscle membranes.

With regard to the Hill slope, 1.0 and 0.97 were obtained for semotiadil and D51-4700 in the cardiac membranes, respectively, and 0.63 and 0.67 in the skeletal muscle membranes, suggesting no apparent allosteric interactions exist between the semotiadil analogs and PN200-110 in the cardiac membranes but negative allosteric interactions operate in the skeletal muscle membranes. The IC_{50} (2.0 μM) and Hill slope (0.63) of semotiadil for the skeletal membranes are close to the reported values in canine skeletal membranes (5.4 μM and 0.57, respectively) [21,22]. As reference, (±)PN200-110 inhibition in the cardiac and skeletal muscle membranes is shown in Fig. 1, where Hill slopes were 1.0 and 0.97, and IC_{50} were 0.4 nM and 2.0 nM, respectively, values which are consistent with previous observations [21,34].

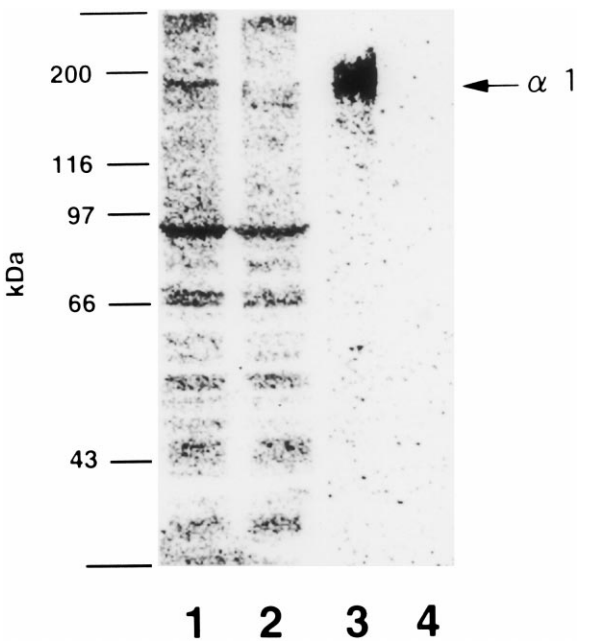

Fig. 2. Photolabeling of cardiac Ca^{2+} channel preparations with [^{3}H]D51-4700. Sarcolemmal-rich membranes from porcine hearts were photolabeled with 200 nM [^{3}H]D51-4700 in the absence (lane 1) or presence (lane 2) of 10 μM semotiadil. Both samples were then solubilized and purified to give the purified samples that were photolabeled in the absence (lane 3) or presence (lane 4) of 10 μM semotiadil. All the samples were analyzed on SDS-PAGE followed by radioluminography. The migration of the α1 subunit and of molecular mass markers (shown in kDa) is indicated.

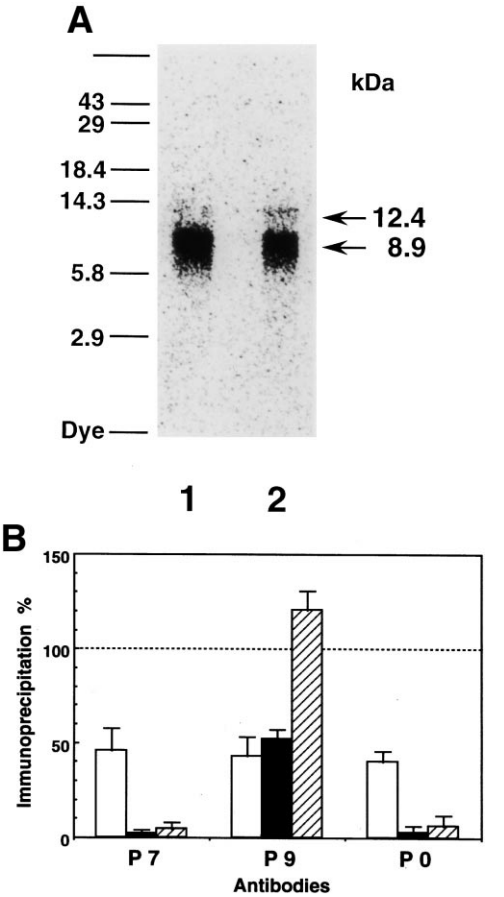

Fig. 3. Analysis of Lys-C digests of photolabeled α1 subunit by SDS-PAGE and immunoprecipitation. A: SDS-PAGE analysis of Lys-C digests of photolabeled α1. [^{3}H]D51-4700-labeled α1 was digested with Lys-C and an aliquot was separated on a Schägger and von Jagow gel (lane 1). Another aliquot was subjected to immunoprecipitation with anti-P9 antibody and the immunoprecipitated fragments were analyzed on the same gel system (lane 2). The photolabeled band was visualized by radioluminography. The migration of prestained molecular mass markers (given in kDa) is indicated. B: Immunoprecipitation of [^{3}H]D51-4700-labeled peptide fragment from Lys-C digests. Photolabeled α1 subunits were digested in the absence (open bars) or presence (filled bars) of Lys-C. Both samples were probed in parallel with the anti-peptide antibodies. The immunoprecipitated percentages are shown and the immunoprecipitated dpms normalized with respect to the dpms immunoprecipitated in non-digested samples (100%) are shown as hatched bars.

3.2. Photolabeling

[^{3}H]D51-4700 photolabeled several polypeptides in the porcine cardiac membranes (Fig. 2, lane 1). However, only the α1 subunit-bound label (180 kDa) of the Ca^{2+} channel was selectively inhibited in the presence of excess semotiadil (lane 2).

Selective labeling was also confirmed when the photolabeled cardiac membrane preparation was solubilized by digitonin and affinity-purified by WGA-Sepharose and antibody column. In the purified sample, a single band of 180 kDa was photolabeled (lane 3) while the labeled band was not observed when photolabeling was done in the presence of excess semotiadil (lane 4).

3.3. Localization

To determine the localization of the photolabeled site within the α1 subunit, we subjected the photolabeled α1 subunit to protease digestion with an endoprotease Lys-C and probed the Lys-C fragment by immunoprecipitation with a series of sequence-directed antibodies (see Section 2) against different regions of α1. The Lys-C digestion of α1 is shown in Fig. 3, resulting in labeled fragments of 8.9 kDa predominantly and 12.4 kDa as a minor product (Fig. 3A, lane 1).

In immunoprecipitation, all three antibodies reacted with cardiac non-digested α1 subunit at 40–45% (Fig. 3B), indicating that the anti-peptide antibodies that were raised against the peptide sequence of rabbit skeletal muscle channels have almost equal potencies to crossreact with the corresponding porcine cardiac channel peptides, even though two peptides (P0 and P7) have one or two different amino acid substitutions. In contrast, immunoprecipitation after Lys-C digestion with the same antibodies revealed that only the anti-P9 antibody directed against sequence 1507–1525 immunoprecipitated the photolabeled fragments while anti-P0 and anti-P7 antibodies did not immunoprecipitate at all (Fig. 3B).

It is of note that about 51% of the α1-associated labeling was associated with the fragment recognized by the anti-1507–1525 antibody, because the immunoprecipitated percentage was calculated as 121% when the radioactivity of the immunoprecipitated fragment was normalized with respect to that in non-digested samples (100%) (Fig. 3B). Similar result have been reported in the photolabeling of skeletal muscle channels with [^{3}H]D51-4700 [23]. This is probably due to the fact that higher reactivity of the anti-peptide antibodies occurs to the peptide fragments than to the non-digested polypeptide α1. Therefore, applied radioactivity was recognized quantitatively by the anti-1507–1525 antibody, strongly suggesting that the labeled site is located solely in a fragment of the 8.9 kDa band. The latter was confirmed by SDS-PAGE analysis of the antibody-bound radioactivity (Fig. 3A, lane 2).

4. Discussion

Binding studies of semotiadil to Ca^{2+} channels in skeletal muscle have been reported [20–22], but no data are available for cardiac preparations. We first tried saturation binding, using varying concentrations of tritiated analogs of semotiadil and D51-4700 to cardiac membranes, but the level of nonspecific binding was too high for analysis. However, inhibition assays of semotiadil and D51-4700 for [^{3}H](+)PN200-110 binding to cardiac membrane preparations were successfully carried out and analyzed. Hill slopes of the binding inhibition were 0.94–1.0 in cardiac channels, indicating no apparent allosteric interaction between PN200-110 (isradipine) and semotiadil. This is in clear contrast to observations in the skeletal channel [20–22], in which semotiadil has a negative allosteric interaction with DHP, PAA and BTZ. The negative allosteric interaction was confirmed in the present work and the Hill slopes obtained were 0.63–0.67, similar to a previous report [21]. In addition, IC_{50} values for binding inhibition of semotiadil and its analog to the cardiac Ca^{2+} channel were 10 times higher than those for skeletal muscle. These results suggest that the semotiadil binding region is differently configured in the Ca^{2+} channels from heart and skeletal muscle.

Photoaffinity labeling and location of the labeled site were employed in determining structural requirements for the different binding in the cardiac channel. The photolabeled site of the cardiac Ca^{2+} channel was located to the single Lys-C-digested peptide fragment of Cys^{1461}–Lys^{1529} (8.9 kDa), as shown in Fig. 4. The peptide was similar to the photolabeled and Lys-C-digested fragment (8.3 kDa) in skeletal muscle Ca^{2+} channel (Fig. 4) which we previously reported [23]. As far as we know of Ca^{2+} antagonists, this is the first example that photolabeled sites are identified in two types of L-type Ca^{2+} channels from different tissues. The present data suggest that the semotiadil binding site is configured with the homologous segment containing transmembrane IVS6 and adjacent extracellular and/or intracellular stretches in both Ca^{2+} channels. However, it must be noted that the sequences are not identical.

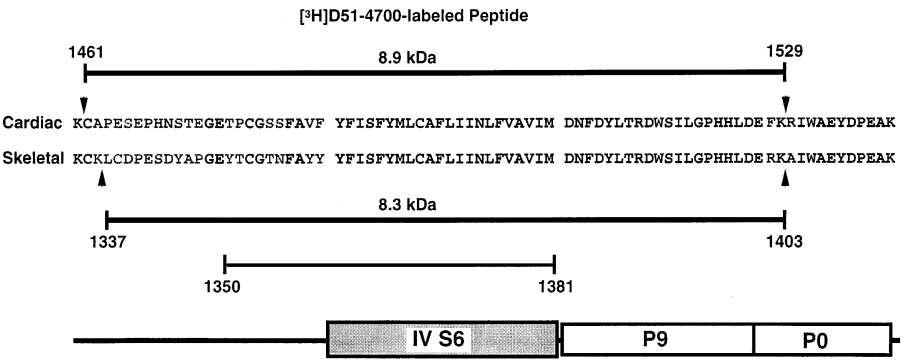

Fig. 4. Comparison of labeled fragment location in the cardiac and skeltal muscle Ca^{2+} channels. Amino acid sequences of the porcine cardiac peptide fragment (8.9 kDa) which is located as [^{3}H]D51-4700-labeled site is shown by comparison with the corresponding fragment in the rabbit skeltal muscle counterpart (8.3 kDa) and its smallest labeled region (1350–1381) identified previously [23]. The sequence of the cardiac peptide (1460–1540) from porcine heart was confirmed to be identical to that from rabbit heart [26] by RT-PCR. Therefore, between two sequneces of the cardiac and skeletal channels, identical and non-identical amino acids are shown in dark and light bold, respectively. A schematic alignment of the transmembrane segment S6 (shadowed) in repeat IV and antibodies (P9 and P0) used is also indicated at the bottom.

The results, taken together, permit us to speculate on the labeling and binding sites as follows. Within the 8.9 kDa fragment, the most probable labeled site is the peptide between Thr^{1475} and Met^{1507} which is the corresponding peptide (Tyr^{1350}–Met^{1381}) identified in the skeletal muscle counterpart [23]. There are six different amino acids in the N-terminal portion. If this is the case, the binding sites for the two channels may consist of the same local segment but different amino acid residues are probably involved in the expression of the different binding characteristics of semotiadil and its photoaffinity analog. The adjacent N-terminal extracellular stretch is another candidate for the labeled site since the sequences are different between the two channels except for two amino acids, Gly-Glu. In this context, it is less probable that the labeled site exists in the intracellular stretch because the amino acid sequence Asp^{1507}–Lys^{1529} in the cardiac fragment is identical to the skeletal muscle sequence except for Phe^{1528}.

Alternatively, it is possible that the binding site is composed of several segments and the identified region by photoaffinity labeling is only a component of the entire binding site. PAA is such an example in which photolabeling occurred in the single peptide containing segment IVS6 with the adjacent extracellular and intracellular stretches [11]. Subsequent site-directed mutagenesis showed that not only IVS6 [12,13] but also IIIS6 appears to determine high affinity binding of a PAA analog [14].

This is the first example of differential binding sites for a particular Ca^{2+} antagonist in two L-type Ca^{2+} channels. Further molecular biological studies are necessary to clarify the semotiadil binding site including whether additional segment(s) is/are required to form the pocket for cardiac and skeletal muscle Ca^{2+} channels.

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