Photochemical Identification of Transmembrane Segment IVS6 as the Binding Region of Semotiadil, a New Modulator for the L-type Voltage-dependent Ca\(^{2+}\) Channel*

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To identify the binding domain of a new Ca\(^{2+}\) antagonist semotiadil on L-type Ca\(^{2+}\) channels from skeletal muscle, photolabeling was carried out by using an azido-derivatized derivative of \(^{3}H\)semotiadil. Photoincorporation was observed in several polypeptides of membrane triad preparations; the only specific photoincorporation was in the \(\alpha\) subunit of the Ca\(^{2+}\) channel. After solubilization and purification, the photolabeled \(\alpha\) subunit was subjected to proteolytic and CNBr cleavage followed by antibody mapping. Specific labeling was associated solely with the region of transmembrane segment S6 in repeat IV. Quantitative immunoprecipitation was found in the tryptic and the Lys-C/Glu-C fragments of 6.6 and 6.1 kDa, respectively. Further CNBr cleavage of the Lys-C digests produced two smaller fragments of 3.4 and 1.8 kDa that were included in the tryptic and Lys-C/Glu-C fragments. The smallest labeled fragments: Tyr\(^{1350}\)-Met\(^{1366}\) and Leu\(^{1367}\)-Met\(^{1381}\) containing IVS6, a possible pore-forming region. The data suggest that semotiadil binds to a region that is overlapped with but not identical to those for phenylalkylamines, dihydropyridines and benzothiazepines. The present study also provides evidence that region IV represents an important component of a binding pocket for Ca\(^{2+}\) antagonists.

Ca\(^{2+}\) antagonists bind with high affinity to L-type Ca\(^{2+}\) channels and block the entry of extracellular Ca\(^{2+}\). Three specific classes of Ca\(^{2+}\) antagonists have been identified and include 1,4-dihydropyridines (DHP), \(^{1}\) phenylalkylamines (PAA), and benzothiazepines (BTZ), which are represented by the parent compounds, nifedipine, verapamil, and diltiazem, respectively. These drugs bind to different sites on the \(\alpha\) subunit of Ca\(^{2+}\) channels (1), and logically explain the well known allosteric interactions with one another (2). Using photoaffinity labeling and antibody mapping techniques, all three drugs have been shown to bind to different regions in more than one motif. Several other Ca\(^{2+}\) antagonists have different chemical structures and somewhat different pharmacological actions than DHP, PAA, and BTZ (3–5). Semotiadil (SD-3211) is a novel Ca\(^{2+}\) antagonist with a unique 1,4-benzothiazine ring structure (3) (Fig. 1).

The benzothiazine ring is homologous to the benzothiazepine ring of diltiazem whereas the ring components of the two drugs might contribute different properties in the action on Ca\(^{2+}\) channels. Studies on structure-function relationships of diltiazem (reviewed in Ref. 6) suggest that the acetoxy and 2-(dimethylamino)ethyl groups play important roles in the calcium antagonistic activity. It is likely that the benzothiazepine ring of diltiazem is a structure on which various side groups can be inserted, which may change the position of the ring in binding and subsequent inhibition of the Ca\(^{2+}\) channel. For example, the hydrophobic 4-methoxophenyl group as well as the acetoxy and 2-(dimethylamino)ethyl groups, probably confer specific activities of diltiazem and other BTZs. In contrast, the calcium antagonist activity of semotiadil depends, in part, on the long side chain of Ar–O–CH\(_2\)CH\(_2\)CH\(_2\)–(NMe)–CH\(_2\)CH\(_2\)–O–Ar which is a structure on which various side groups can be inserted, which may change the position of the ring in binding and subsequent inhibition of the Ca\(^{2+}\) channel.
increases the dissociation rate of $[^3H](+\cdot)PN200–110$, $[^3H]$diltiazem and $[^3H]$verapamil binding sites (11–13). These results suggest that semotiadil has a strong allosteric interaction with the three classes of $Ca^{2+}$ antagonists, as exemplified by differential displacement of $[^3H]PN200–110$, $[^3H]$diltiazem, and $[^3H]$verapamil from their specific sites on the $Ca^{2+}$ channels (11–13).

Localization of the semotiadil binding site would provide information about a putative new class of $Ca^{2+}$ antagonists but more importantly might uncover overlapping binding region(s), if any, with conventional $Ca^{2+}$ antagonists. The binding sites for DHP, PAA, and BTZ have been localized by photoaffinity labeling of $Ca^{2+}$ channels followed by defined proteolysis and antibody mapping using sequence-directed antibodies (14–18). By comparing the results of the latter, with those derived from mutagenesis experiments (19–25), one can demonstrate that sequence stretches photolabeled by DHP, PAA, and BTZ indeed contain amino acid residues that directly participate in binding. However, some recent mutagenesis experiments (26–28) have revealed sites that are not labeled by photoligands. As an initial work to identify the site for semotiadil, we employed techniques of photoaffinity labeling of $Ca^{2+}$ channels isolated from rabbit skeletal muscles with $[^3H]$D51–4700, an azidoephilic derivative of $[^3H]$semotiadil (29), and the localizing of the site(s) of photolabeling and comparing with those for DHP, PAA, and BTZ.

**EXPERIMENTAL PROCEDURES**

**Materials**—$[^3H]$D51–4700 (77.6 Ci/mmol) was synthesized as described (29). Semotiadil was obtained from Daiichii Pharmaceutical Co., Ltd. Enzymes and chemicals were obtained from the following sources: N-p-toluenesulfonyl-l-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin from bovine pancreas) from Worthington; endoprotease Glu-C (0.5 mg/ml) and digentin from Achromobacter lyticus (30). The sample was dialyzed against 1 mM Tris-HCl (pH 9.0) containing 0.05% (w/v) SDS (final volume of 0.1 ml). After incubation at room temperature for 30 min, iodoacetic acid was added to a final concentration of 84 mM. After incubation for 1 h, the photolabeled $\alpha_1$ subunits were further purified by gel permeation liquid chromatography as described (14). Fractions corresponding to the $\alpha_1$ subunit were pooled, lyophilized, and stored at $-30^\circ$C until use.

**Peptide Synthesis and Antibody Production**—Polyclonal antibodies were raised in rabbits against synthetic peptides corresponding to particular regions of the skeletal muscle $\alpha_1$ subunit sequence (30): 1320–1332 (anti-1320–1332), 1338–1351 (anti-1338–1351), 1382–1400 plus the N-terminal Gly-Cys (anti-1382–1400), 1401–1414 plus C-terminal Cys-Gly (anti-1401–1414). The peptide was conjugated to bovine serum albumin or bovine thyroglobulin via a cysteine residue using N-hydroxysuccinimidyl maleimidobenzoate. Japanese white rabbits were immunized with the conjugate emulsified in Freund’s complete adjuvant. After 3 weeks, the immunizaton was repeated 5 times at 2-wk intervals with the conjugate in Freund’s incomplete adjuvant.

**Membrane Preparation**—Triad membranes were isolated from rabbit skeletal muscle as described by Mitchell et al. (31).

**Photoaffinity Labeling and Purification of Rabbit Skeletal $Ca^{2+}$ Channels**—Rabbit triad membranes (300 pmol of $[^3H](+\cdot)PN200–110$ binding sites, 20 mg of proteins) were incubated with 100 nM $[^3H]$D51–4700 in 10 ml of binding buffer (25 mM Tris-HCl (pH 7.2), 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml pepstatin A, 1 $\mu$g/ml leupeptin, 10 $\mu$g/ml soybean trypsin inhibitor) in the presence and absence of 10 $\mu$M 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 watt black light/white lamp (Ultra-Violet Products, Inc., San Gabriel, CA) at distance of 10 cm. After photolysis, the $[^3H]$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 (30). The sample was dialyzed against 1 mM Tris-HCl (pH 7.3) and lyophilized.

**Reductive Carboxymethylation and Gel Permeation High Pressure Liquid Chromatography**—The photolabeled and lyophilized protein was resuspended in 0.1 $\times$ Tris-HCl (pH 8.0), 1% (v/v) 2-mercaptoethanol, 1.5% (w/v) SDS (final volume of 0.3 ml). After incubation at room temperature for 30 min, iodoacetic acid was added to a final concentration of 84 mM. After incubation for 1 h, the photolabeled $\alpha_1$ subunits were further purified by gel permeation liquid chromatography as described (14). Fractions corresponding to the $\alpha_1$ subunit were pooled, lyophilized, and stored at $-30^\circ$C until use.

**Proteolytic and CNBr Cleavage of $[^3H]$D51–4700-labeled $\alpha_1$ Subunits**—The photolabeled $\alpha_1$ subunit was dissolved in deionized water (0.5 ml) and dialyzed against 6 $\times$ urea as described (14), followed by dialysis against 0.01% Triton X-100 for 6 h. The sample was digested with Lys-C (50 $\mu$g/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 $\mu$l) at 37 °C for 6 h. For trypsin digestion, the sample was incubated with TPCK-trypsin (100 $\mu$g/ml) in 37 °C for 12 h in 50 mM Tris-HCl (pH 8.0) containing 0.01% (v/v) Triton X-100 and 2 mM CaCl$_2$. The reaction was stopped by heating at 90 °C for 3 min. Prior to Lys-C/Glu-C digestion and CNBr cleavage, Lys-C digests were dialyzed against H$_2$O for 6 h using a microdialyzer apparatus with a 1 kDa cut-off dialysis tube (Spectra/Por 6, Spectrum). For Lys-C/Glu-C digestion, the dialyzed sample was incubated with Glu-C (0.5 mg/ml) in 50 mM sodium phosphate buffer (pH 7.8) containing 0.05% (w/v) SDS for 12 h at 37 °C. For CNBr cleavage, the dialyzed sample was lyophilized and then incubated with CNBr (5 mg/ml) in 70% (v/v) formic acid for 12 h at 37 °C. After incubation, the mixture was lyophilized.

**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% (w/v) Triton X-100 and 1 mg/ml bovine serum albumin) for 2 h at 4 °C. The gel was washed with the ice-cold buffer A before addition of...
FIG. 2. Photolabeling of Ca\(^{2+}\) channel preparations with \([\text{H}]\text{D}51–4700\). Triad membranes from rabbit skeletal muscles (2 mg/ml) were photolabeled with 100 nM \([\text{H}]\text{D}51–4700\) in the absence (lane 1) and presence (lane 2) of 10 μM semotiadil. Aliquots (20 μl) of the photolabeled mixture were spun down, and the pellet was solubilized by the sampling buffer for SDS-PAGE and analyzed on a 8% polyacrylamide gel followed by radioluminography. The photolabeled samples were also solubilized with 1% (w/v) digitonin and partially purified by WGA-Sepharose 4B. The purified samples (2.5 μg) that were photolabeled in the absence (lane 3) and presence (lane 4) of 10 μM semotiadil were similarly analyzed on the SDS-PAGE followed by radioluminography. The migration of the \(\alpha_1\) subunit and of molecular mass markers (shown in kDa) is indicated.

FIG. 3. Lys-C digestion of photolabeled \(\alpha_1\) subunits. A, \([\text{H}]\text{D}51–4700\)-labeled \(\alpha_1\) was digested with Lys-C (50 μg/ml, 37 °C, 6 h) and 30-μl aliquots were separated on a Schägger and von Jagow (34) gel (lane 1). A 30-μl aliquot was also subjected to immunoprecipitation with anti-(1338–1351) (lane 2) and anti-(1382–1400) (lane 3). The photolabeled band was visualized by radioluminography. The arrow indicates the 8.3-kDa fragment. The migration of prestained molecular mass markers (given in kDa) is indicated. In the separate experiments, 92 ± 4% (approximately 850 dpm) of the radioactivity applied on the gel was detected in the band centered on 8.3 kDa by liquid scintillation counting of 3-mm gel slices (not shown). Five runs were carried out. B, Immunoprecipitation of \([\text{H}]\text{D}51–4700\)-labeled peptide fragment from Lys-C digests. Photolabeled \(\alpha_1\) subunits were digested in the absence (control) or presence of Lys-C. Both samples were probed in parallel with the antibodies against the indicated \(\alpha_1\) peptides. The immunoprecipitated percentages (immunoprecipitated dpm per applied dpm) are shown as filled bars and the immunoprecipitated dpm were normalized with respect to the dpm immunoprecipitated in nondigested samples (100%) are shown as open bars. Means ± S.D. are given for \(n = 3\).

Results

Specific Photoincorporation of the 170-kDa \(\alpha_1\) Subunit of Rabbit Skeletal Muscle Tubules—The synthesis and pharmacological characterization of the photoaffinity ligand \([\text{H}]\text{D}51–4700\) have been described (29). \([\text{H}]\text{D}51–4700\) photolabeled several polypeptides as shown in Fig. 2 (lane 1). However, only the \(\alpha_1\) subunit bound label (170 kDa) of the Ca\(^{2+}\) channel was selectively inhibited in the presence of excess semotiadil (lane 2). The selective labeling was also confirmed when the photolabeled triad preparation was solubilized by digitonin and purified by a WGA-Sepharose column (30). In the purified sample, a single band of 170 kDa was photolabeled (lane 3), whereas the labeled band was not observed when photolabeling was done in the presence of excess semotiadil (lane 4).

[\([\text{H}]\text{D}51–4700\) labeling occurs only within repeat IV—To determine the localization of photolabeled site within the \(\alpha_1\) subunit, we first subjected the photolabeled \(\alpha_1\) 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 “Experimental Procedures”) against different regions of \(\alpha_1\). The Lys-C digestion of \(\alpha_1\) is shown in Fig. 3, resulting in a labeled fragment of 8.3 ± 0.7 kDa (\(n = 5\)) (Fig. 3A, lane 1). The fragment contained 92 ± 4% (\(n = 5\)) of the \(\alpha_1\)-associated radioactivity as determined by gel slicing (not shown). Immunoprecipitation with sequence-directed antibodies revealed that only two antibodies directed...
fragments. The migration of prestained molecular mass markers (given arrows visualized by radioluminography. The precipitation with anti-(1338–1351) (1338–1351) and anti-(1382–1400) suggesting that both of the radiolabeled radioactivities immunoprecipitated in nondigested samples (1382–1400) were normalized with respect to the bands for 8.3- and 6.6-kDa by liquid scintillation counting of 3-mm gel slices. (Resolution was not enough in the sliced gels.) Three runs were carried out. B, immunoprecipitation of [3H]D51–4700-labeled peptide fragments from TPCCK-trypsin digests. The immunoprecipitated percentages (immunoprecipitated dpm per applied dpm) by anti-(1338–1351) and anti-(1382–1400) are shown. Means ± S.D. are given for n = 3.

against epitopes located near segment S6 in repeat IV (anti-(1338–1351) and anti-(1382–1400), see Fig. 7) immunoprecipitated the photolabeled fragments, whereas anti-(1320–1332) and anti-(1401–1414) did not immunoprecipitate at all (Fig. 3B). Other antibodies against repeat I, repeat III, and repeat IV efficiently immunoprecipitated the nondigested labeled α1 but did not immunoprecipitate Lys-C fragments (not shown). About 56–68 and 57–75% of the α1-associated labeling were associated with a fragment recognized by anti-(1338–1351) and anti-(1382–1400), respectively (Fig. 3B). After the immunoprecipitated radioactivities were normalized with respect to the radioactivities immunoprecipitated in nondigested samples (100%), the values calculated were 125–147 and 124–163%, respectively (Fig. 3B). The reason why the calculated values were over 100% will be discussed later (see “Discussion”). The radioactivity applied was recognized quantitatively by anti-(1338–1351) and anti-(1382–1400) suggesting that both of the antibodies were immunoprecipitating the same 8.3-kDa band. This was confirmed by SDS-PAGE analysis of the antibody bound radioactivity (Fig. 3A, lane 2 and 3). Since the extracellular α1 (1338–1351) or intracellular α1 (1382–1400) epitope is located within a single Lys-C fragment that contains IVS6 and intracellular residues, or IVS6 and extracellular residues, respectively, the 8.3-kDa fragment represents the correct digested product at Lys1336 and Lys1403 (calculated mass 7.9 kDa, see Fig. 7).

[3H]D51–4700 Labeling Is Located in Tryptic Fragments Containing the S6 Segment in Repeat IV—Since the Lys-C fragment contains cleavable sites by trypsin, the photolabeled α1 subunits were digested with TPCCK-trypsin to refine the photolabeled sites. SDS-PAGE revealed two smaller labeled fragments with apparent molecular masses of 8.3 ± 0.8 (n = 3) and 6.6 ± 0.7 kDa (n = 3). A radioluminogram of a gel where two peaks are clearly separated is shown in Fig. 4A. 84 ± 8% of the α1-associated radioactive recovery was observed in these peaks and no other smaller fragments were observed as determined by gel slicing (not shown). Location of the photolabeled tryptic fragments was assessed by immunoprecipitation using anti-(1338–1351) and anti-(1382–1400). About 53–61% of the α1-associated labeling were associated with fragments recognized by anti-(1338–1351) (Fig. 4B). The immunoprecipitated peptides were 8.3 and 6.6 kDa, determined by SDS-PAGE analysis (Fig. 4A, lane 2). The 6.6-kDa peptide was immunoprecipitated to a greater extent than the 8.3-kDa peptide, which is in accordance with the fact that the 6.6-kDa band was the major labeled peptide (Fig. 4A). Therefore, both peptides must contain the full epitope sequence of anti-(1338–1351) and the 6.6-kDa peptide must be the smallest labeled peptide obtained by trypsin digestion.

In contrast, immunoprecipitation by anti-(1382–1400) decreased markedly to 12–18% (Fig. 4B) compared with the results obtained with the Lys-C fragment. The epitope of anti-(1382–1400) contains an arginine residue at 1389 that was cleavable by trypsin. Therefore, the major labeled fragment (6.6 kDa) must be generated by trypsin cleavage at Arg1389 and is not recognized by anti-(1382–1400) (see Fig. 7). The antibody only recognizes the minor labeled fragment (8.3 kDa) that is a partially trypsin-digested product containing the epitope region (1382–1400).

Since no smaller fragments than 8.3 and 6.6 kDa were obtained, the 6.6 kDa must be the smallest labeled peptide obtained by trypsin digestion. The peptide contains the epitope of anti-(1338–1351) but loses the epitope of anti-(1382–1400). The 6.6-kDa labeled peptide, therefore, is derived by trypsin cleavage at Lys1336 and Arg1403 (calculated molecular mass as 6.1 kDa) and contains IVS6 together with adjacent extracellular and cytoplasmic amino acid residues.

Isolation and Characterization of Smaller Photolabeled Fragments by Glu-C Digestion—Since the Lys-C fragment also contains potential cleavable sites by Glu-C, the photolabeled Lys-C fragment was subsequently digested with endoproteinase Glu-C to further restrict the photolabeled sites. As shown in Fig. 5A, a radioluminogram of a gel revealed two smaller labeled fragments with apparent molecular masses of 7.8 ± 0.9 (n = 3) and 6.1 ± 0.7 kDa (n = 3). The α1-associated radioactivity was recovered in 86 ± 7% in these peaks and no other peaks.
3.4-kDa fragments. The migration of prestained molecular mass markers (given in kDa) is indicated.

In immunoprecipitation experiments, anti-(1382–1400) retained its binding activity (62%) to the Glu-C digested fragments whereas anti-(1338–1351) showed only 20% immunoprecipitation of the digests (Fig. 5B). Since the epitope of anti-(1338–1351) contains three glutamic acid residues (at positions 1341, 1348 and 1349) that are susceptible to Glu-C cleavage, the low efficiency in immunoprecipitation by anti-(1338–1351) must result from the cleavage. The photolabeled and immunoprecipitated peptides by anti-(1382–1400) were analyzed by SDS-PAGE (Fig. 6A, lane 2). The 6.1-kDa photolabeled peptide was observed in addition to a small portion of 7.8-kDa fragments, indicating that the 6.1-kDa peptide was the smallest labeled fragment after successive digestion with Lys-C and Glu-C. According to our estimation of molecular mass, the cleavage site by Glu-C most likely corresponds to Glu-1349 (calculated molecular mass 6.2 kDa). Fig. 7 shows the position of the smallest photolabeled fragment by Lys-C/Glu-C digests within the linear alignment near IVS6 segment.

**Isolation and Characterization of Smaller Photolabeled Fragments by CNBr Cleavage**—Since the Lys-C fragment contains two methionine residues, the photolabeled Lys-C fragment was subsequently treated with CNBr to further restrict the photolabeled sites. As shown in Fig. 6A, a radioluminogram of a gel revealed three smaller labeled fragments with apparent molecular masses of 5.7 ± 0.6 (n = 3), 3.4 ± 0.4 (n = 3), and 1.8 ± 0.3 kDa (n = 3). During the incubation with CNBr in 70% formic acid, almost 70% of the photolabeled radioactivity was liberated and migrated to the dye front position on SDS-PAGE (Fig. 6B). However, the liberated radioactivity was not blotted on the polyvinylidene difluoride membrane sheet, and therefore it did not interfere with the analysis of newly generated labeled fragments in the radioluminogram (Fig. 6A). In the immunoprecipitation experiments, anti-(1338–1351) showed apparent binding activity (10 ± 9%, n = 3) to the total radioactivity applied after CNBr cleavage, whereas anti-(1382–1400) did not immunoprecipitate at all. As the radioactivity associated with peptide fragments was only 30% of the radioactivity in the applied sample, the immunoprecipitated value of 10% can be corrected to 33%. This value is further corrected to 73% after normalization with respect to immunoprecipitation avidity of anti-(1338–1351) in uncleaved α1 subunits (45%).

The photolabeled and immunoprecipitated peptides by anti-(1338–1351) were analyzed by SDS-PAGE (Fig. 6A, lane 2). The 3.4 and 5.7-kDa photolabeled peptides were observed, but the 1.8-kDa fragment was not immunoprecipitated. The results indicate that the 3.4- and 5.7-kDa fragments contain the epitope of anti-(1338–1351). Therefore, we assign the labeled 3.4-kDa fragment to Leu1357–Met1360 (calculated molecular mass 3.6 kDa) and the 5.7-kDa fragment to Leu1337–Met1341 (calculated molecular mass 5.3 kDa) that is a partially cleaved product at Met1341 but not cleaved at Met1360. On the other hand, the nonimmunoprecipitated labeled fragment of 1.8 kDa must be Leu1367–Met1381 (calculated molecular mass 1.7 kDa) that contains no sequence for the epitope of anti-(1338–1351). The smallest labeled fragments are 3.6 kDa (Leu1337–Met1360) and 1.8 kDa (Leu1367–Met1381). Fig. 7 shows the position of these photolabeled fragments by CNBr cleavage within the linear alignment near segment IVS6.

**DISCUSSION**

*Semotiadil Receptor Site of the α1 Subunit*—[3H]D51–4700, a photoaffinity probe of semotiadil, selectively labeled the α1 subunit of Ca2+ channels in skeletal triad membranes. In the absence of unlabeled semotiadil, the probe labeled several polypeptides including the α1 subunit. This may explain the observation that reversible binding of [3H]D51–4700 to triad membrane preparations is rather difficult to show due to the high level of nonspecific binding (not shown). However, the photoincorporation to the α1 subunit occurred in a specific manner since the Ca2+ channels purified by WGA column showed a single photolabeled band of 170 kDa, and the label was totally blocked by excess unlabeled semotiadil. The specifically photolabeled site was localized within the α1 subunit by an antibody mapping method employed previously for the DHPR, PAA-, and BTZ-binding domains (14–18). As shown in the results of Lys-C digestion, we observed that the normalized values of the immunoprecipitated percentage of the protease-digested fragment gave more than 100% with respect to those of the nondigested samples. Similar results were reported in the literature (16) where the labeled site was localized to a single peptide fragment. This is probably due to the fact that higher reactivity of the anti-peptide antibody occurs to the
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peptide fragment rather than to the nongested polypeptide $\alpha_1$.

Only a single labeled fragment of 8.3 kDa was obtained by Lys-C digestion of the $[^3H]D51–4700$ labeled $\alpha_1$ subunit. From searching the overlapped peptide sequences obtained by the proteolytic digestion and CNBr cleavage, the smallest labeled fragments were Tyrr$_{1350}$-Met$_{1366}$ and subsequently Leu$_{1367}$-Met$_{1381}$. The peptide Tyrr$_{1350}$-Met$_{1366}$ contains the N-terminal half of the transmembrane segment S6 of repeat IV together with a short extracellular stretch, and the peptide Leu$_{1367}$-Met$_{1381}$ contains the subsequent C-terminal half of the transmembrane segment S6 of repeat IV.

Implication of the Semotiadil Binding Site Compared with Other Ca$^{2+}$ Antagonists—The labeled fragments by $[^3H]D51–4700$ are identified as Tyrr$_{1350}$-Met$_{1366}$ and Leu$_{1367}$-Met$_{1381}$ in IVS6 after CNBr cleavage. They are included in the Glu-C fragment of Tyr$_{1350}$-Trp$_{1391}$, which was identified as the labeled peptide by $[^3H]LU49888$ (16), a photoaffinity probe of PAA, but the intracellular region of Asp$_{1382}$-Trp$_{1391}$ is not included in the $[^3H]D51–4700$ labeled fragments. Since smaller $[^3H]LU49888$ fragments than those generated by Glu-C digestion have not been mapped, we cannot exclude the possibility that $[^3H]LU49888$ did not label the intracellular region of Asp$_{1382}$-Trp$_{1391}$. However, semotiadil does not compete with the binding of PAA but rather allosterically inhibits binding or vice versa (11, 12). This suggests that the binding site for semotiadil is similar but not identical to that for PAA. The present results are consistent with this interpretation.

The two labeled fragments by $[^3H]D51–4700$ are not only overlapped with the $[^3H]LU49888$ labeled site but also are part of the labeled regions by DHP (14, 15) and BTZ (17, 18). The association of the newly identified semotiadil site with those of the three typical Ca$^{2+}$ antagonists (DHP, BTZ, and PAA) within the pore-forming regions of the channel allows allosteric interactions among semotiadil and these drugs. Although a few reports are available concerning the pharmacological interac-

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Photochemical Identification of Transmembrane Segment IVS6 as the Binding Region of Semotiadil, a New Modulator for the L-type Voltage-dependent Ca\textsuperscript{2+} Channel
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