

PHOTOCHEMICAL IDENTIFICATION OF THE BINDING REGION FOR (*S*)-SEMOTIADIL ON SODIUM CHANNELS: COMPARISON WITH THAT FOR (*R*)-SEMOTIADIL ON SKELETAL MUSCLE CALCIUM CHANNEL[¶]

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Abstract - To clarify the recognition site of (*S*)-semotiadil in the Na⁺ channels, we employed photoaffinity labeling with (*S*)-[³H]-D51-4700, an azidophenyl derivative of (*S*)-semotiadil, to Na⁺ channel preparations from rat brains and porcine hearts, and the results were compared with those by (*R*)-enantiomer to skeletal muscle Ca²⁺ channel. Protease-digestion of the photolabeled Na⁺ channels followed by quantitative immunoprecipitation assay using a site-directed antibody, revealed that 10- and 13- kDa fragments of brain and cardiac Na⁺ channels, respectively, were located within the regions including IVS6. These regions are corresponding to the 8.3 kDa fragment photolabeled by (*R*)-[³H]-D51-4700 in the skeletal muscle Ca²⁺ channel. Interestingly, however, the photoincorporation of (*S*)-[³H]-D51-4700 into the Na⁺ channel was 2-3 times higher than that of (*R*)-isomer; and *visa versa* in the Ca²⁺ channels.

INTRODUCTION

SD-3212 is a (*S*)-stereoisomer of semotiadil fumarate (SD-3211) that is a recently developed Ca²⁺ antagonist. This agent is reported to have anti-arrhythmic properties.^{1, 2} Anti-arrhythmic drugs are classified into four groups, at least; Na⁺ channel blockers (class I), β-blockers (class II), K⁺ channel blockers (class III), and Ca²⁺ channel blockers (class IV). The anti-arrhythmic potency of (*S*)-semotiadil

[¶] This article is dedicated to Professor Yuichi Kanaoka on the occasion of his 75th birthday, with sincere gratitude for introducing us in this fascinating research field.

(SD-3212) is appreciably higher than that of (*R*)-semotiadil (SD-3211) in animal models.^{1,3,4} Subsequent electrophysiological studies show that (*S*)-semotiadil has substantially higher selectivity for Na⁺ channel than Ca²⁺ channel,^{1,4} although the selectivity is not so sharp.

Photoaffinity labeling is a powerful method to determine the ligand binding sites within the primary structures of target macromolecules.⁵ Among wide applications of this technique, we previously identified the binding site of (*R*)-semotiadil in the skeletal muscle⁶ and cardiac⁷ L-type Ca²⁺ channels as the region containing segment IVS6 using photoactivatable analog of (*R*)-semotiadil. We also compared the (*R*)-semotiadil binding site with those for well-known Ca²⁺ antagonists, 1,4-dihydropyridines (DHP), phenylalkylamines (PAA), and benzothiazepines (BTZ), since the binding sites of Ca²⁺ antagonists have been identified by photoaffinity labeling and site-directed mutagenesis.⁸⁻¹² Interestingly, the binding sites are largely overlapped each other in the Ca²⁺ channels, although each Ca²⁺ antagonist shows somewhat different pharmacological action.¹³⁻¹⁵

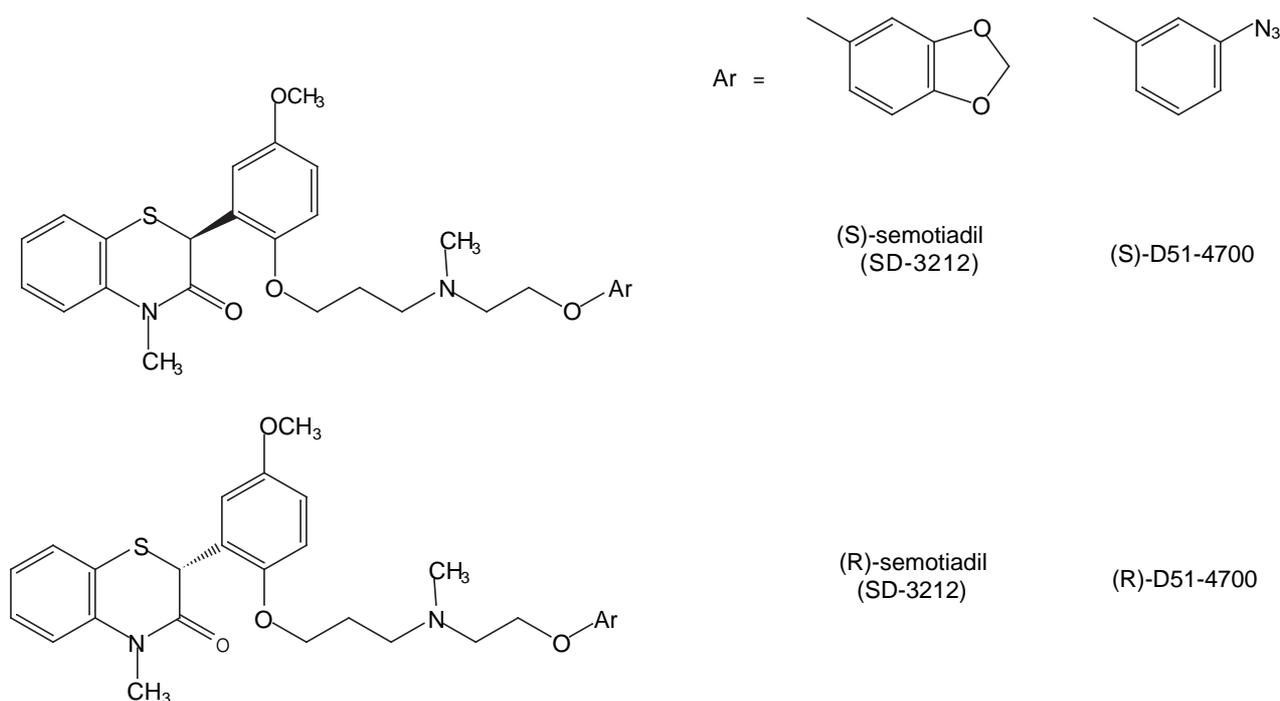


Figure 1. Structures of (*S*)- and (*R*)-semotiadils and their photosensitive derivatives.

For the binding site of (*S*)-semotiadil, however, no data are available at molecular level. We plan to reveal it, because primary structures of Na⁺ channels from several sources are known since the Na⁺ channel from electric eel was first elucidated,¹⁶ and photoaffinity labeling is applicable to the channel molecule (for example, ref. 17 and cited therein). Here we show the results that identify photochemically the binding region of (*S*)-semotiadil in brain and cardiac Na⁺ channels and compare it with that for (*R*)-semotiadil in L-type Ca²⁺ channels we reported previously.^{6,7} We also show and discuss the apparent

enantioselectivity between (*S*)- and (*R*)-semotiadil for Na⁺- and Ca²⁺- channel, respectively, by comparative photolabeling.

RESULTS

Specific Photoincorporation into the 260 kDa α -Subunit of Na⁺ Channel 1 from Rat Brain Synaptosomes – (*S*)-[³H]-D51-4700 photolabeled several polypeptides (Figure 2A, lane 1) including α -subunit (lane 2). The labeling into α -subunit was also confirmed in the purified sample. The photolabeled synaptosome was solubilized by Triton X-100 and then α -subunit was purified by a DEAE-Sephadex and a WGA-Sephadex column successively. A single band of 260 kDa was photolabeled (lane 3), which was recognized by an anti-PCS-1 antibody (lane 4).

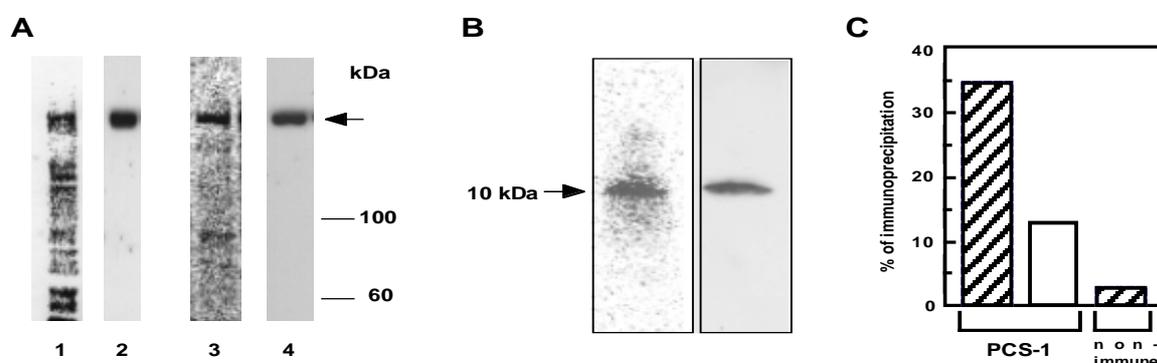


Figure 2. Photolabeling with (*S*)-[³H]-D51-4700 (lanes 1 and 3) and Western blot (lanes 2 and 4) of rat brain synaptosomes before and after purification (A), and Lys-C digestion of photolabeled α -subunit of rat brain Na⁺ channel (B). Immunoprecipitation of the Lys-C digest (C).

(S)-[³H]-D51-4700 Labeling Occurs within the Fragment Adjacent to Segment IVS6 - To determine the localization of photolabeled site within the α -subunit, we subjected the photolabeled α -subunit to the protease digestion with an endoprotease Lys-C and probed the Lys-C fragment by immunoprecipitation with a sequence-directed antibody PCS-1 (see EXPERIMENTAL) against a particular region (1791-1804) of the α -subunit. The Lys-C digestion of α -subunit is shown in Figure 3, resulting in a labeled fragment of 10 kDa (*n* = 3) (Figure 2B, left). The antibody PCS-1 that is directed against epitope located just after the segment S6 in repeat IV (see Figure 5) immunoprecipitated the photolabeled fragment (Figure 2B, right). About 34% of the α -subunit-associated labeling were associated with fragment recognized by antibody PCS-1 (Figure 2C). After immunoprecipitated radioactivity was normalized with respect to the radioactivity immunoprecipitated in non-digested samples (100%), the value was calculated as 300%. The reason why the calculated value was over 100% will be discussed later (see DISCUSSION).

Similar Region of Cardiac Na⁺ Channel was Photolabeled with (S)-[³H]-D51-4700 – When (*S*)-[³H]-D51-4700 was applied to the photolabeling of sarcolemmal fractions from porcine hearts, no selective

photoincorporation into the Na⁺ channel was observed, possibly due to the low density of Na⁺ channel in the sarcolemmal preparations. However, photoincorporation was evident into the α -subunit of cardiac Na⁺ channel, although several other polypeptides were photolabeled, when the solubilized and partially purified Na⁺ channel was used for photolabeling (Figure 3A). After Lys-C digestion of the photolabeled cardiac Na⁺ channel, the digest was subjected to the immunoprecipitation by antibody PCS-1, followed by SDS-PAGE analysis of the immunoprecipitates. As shown in Figure 3B, 17 kDa (major) and 13 kDa (minor) fragments were observed as photolabeled and immunoprecipitated fragments.

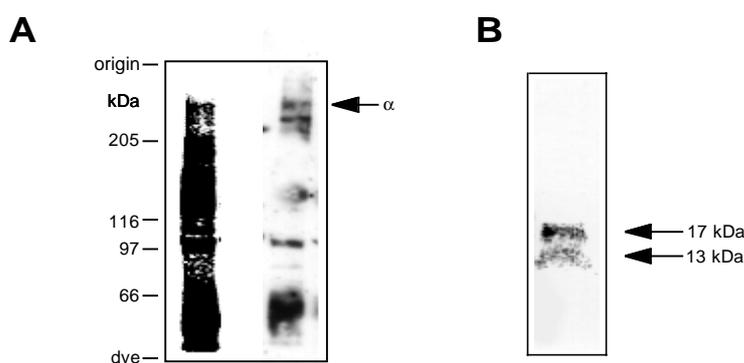


Figure 3. Photolabeling and Western blot of partially purified Na⁺ channel from porcine hearts (A), and immunoprecipitated Lys-C digest of the photolabeled cardiac Na⁺ channel (B).

Effect of Flecanide on Photoincorporation of (S)-[³H]-D51-4700 into Na⁺ Channel - To characterize the binding region of (S)-[³H]-D51-4700 in comparison of anti-arrhythmic agent in the Na⁺ channel, we carried out the photolabeling of rat brain synaptosome in the presence and absence of flecanide and compared. In the presence of 0.5 mM flecanide, photoincorporation of (S)-[³H]-D51-4700 was markedly reduced, nearly close to the level in the presence of (S)-semotiadil (Figure 4A).

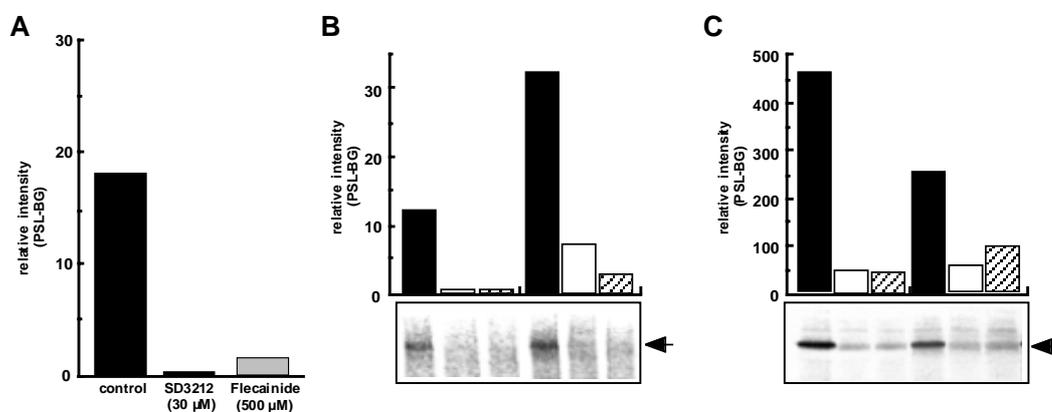


Figure 4. Effects of some drugs on photolabeling with [³H]-D51-4700. **A:** Photolabeling of Na⁺ channel by (S)-[³H]-D51-4700 in the presence or absence of (S)-semotiadil (30 μM) or flecanide (0.5 mM). **B:** Comparative photolabeling of Na⁺ channel by (R)- and (S)-[³H]-D51-4700 in the presence or absence of (R)- and (S)-semotiadil. **C:** Comparative photolabeling of Ca²⁺ channel by (R)- and (S)-[³H]-D51-4700 in the presence or absence of (R)- and (S)-semotiadil.

Comparative Photolabeling of Na⁺ Channel and Ca²⁺ Channel with (S)- and (R)-[³H]-D51-4700 - In order to investigate the preferential binding of semotiadil enantiomer with the respect of binding site structures, photolabeling of Na⁺ channel and Ca²⁺ channel with an enantio pair of [³H]-D51-4700 was performed and compared. Photolabeling of each channel preparation was also conducted in the presence of excess amount of unlabeled enantio pair of semotiadil. As shown in Figure 4B, Na⁺ channel was photolabeled 3-times more efficiently by (S)-isomer of [³H]-D51-4700 (100 nM, *lane 1'*) than (R)-isomer (100 nM, *lane 1*). In addition, the (S)-[³H]-D51-4700 photoincorporation was protected more effectively by addition of excess amount of (S)-semotiadil (*lanes 3 and 3'*) than (R)-semotiadil (*lanes 2 and 2'*). By contrast, Ca²⁺ channel was photolabeled more efficiently by (R)-isomer of [³H]-D51-4700 (*lane 1*) than (S)-isomer (*lane 1'*) at the same concentration (Figure 4C). Addition of (R)-semotiadil protected the photoincorporation of [³H]-D51-4700 into the Ca²⁺ channel (*lanes 2 and 2'*) similarly to or more effectively than (S)-semotiadil (*lanes 3 and 3'*).

DISCUSSION

1. Comparison of photolabeling and the labeled sites between brain and cardiac Na⁺ channels.

(1) Photoincorporation of (S)-[³H]-D51-4700 into Na⁺ channel protein (~260 kDa) was apparent for rat brain synaptosomes. For the porcine cardiac SL membranes, however, the photoincorporation was not so obvious (data not shown), mainly due to less abundance of Na⁺ channels in hearts than those in brain.¹⁸ The photoincorporation into the cardiac Na⁺ channel was confirmed only after purification, as shown in Figure 3A, which also supports suitability of the interpretation described above. We tested the reversible binding assay of (S)-[³H]-D51-4700 using brain synaptosome and cardiac SL membranes. But we were not able to observe significant specific binding, because the nonspecific binding was at quite high level. Similar results had been observed when (R)-[³H]-D51-4700 was applied to the L-type Ca²⁺ channel.⁶

(2) We identified that the photolabeled region of (S)-[³H]-D51-4700 in brain Na⁺ channel was located in a Lys-C fragment of 10 kDa that contains PCS-1 sequence and IVS6, as shown in Figure 5. As shown in the RESULTS, we observed that the normalized value of the immunoprecipitated percentage of the Lys-C-digested fragment gave more than 100% with respect that of the non-digested sample. Similar results were obtained by us^{6,8} and others.⁹ The observation is probably due to the fact that higher reactivity of the anti-peptide antibodies occur to the peptide fragments rather than to the non-digested polypeptides. In the cardiac Na⁺ channel, the photolabeled region was located similar region of 13- or 17-kDa fragment containing PCS-1 sequence and IVS6. The 17 kDa fragment is considered as a precursor of the 13 kDa to be digested by Lys-C (see Figure 5). Consequently, the difference in size of the labeled fragments (10 and 13-kDa) for the brain and cardiac Na⁺ channels, respectively, were simply explained by different location of Lys residues that were cleaved by Lys-C (Figure 5).

2. Enantioselective photolabeling of (S)- and (R)-D51-4700 to Na⁺- and Ca²⁺-channels, respectively.

Enantioselective binding of (S)- and (R)-D51-4700 to Na⁺ - and Ca²⁺ -channels, respectively, was demonstrated by the present photoaffinity labeling study. The demonstration is worth pointing out, because the discrimination of two enantiomers was unsuccessful by reversible binding assay, as described

above. To inspect what region in two channels confers the enantioselectivity for two ligands, we compare amino acid sequence of the two peptide fragments identified by the photolabeling. As shown in Figure 6, the sequence homology was significantly high, suggesting two possibilities for the enantioselectivity: (1) this region is not a sole determinant for the selectivity and other regions composed of different segments

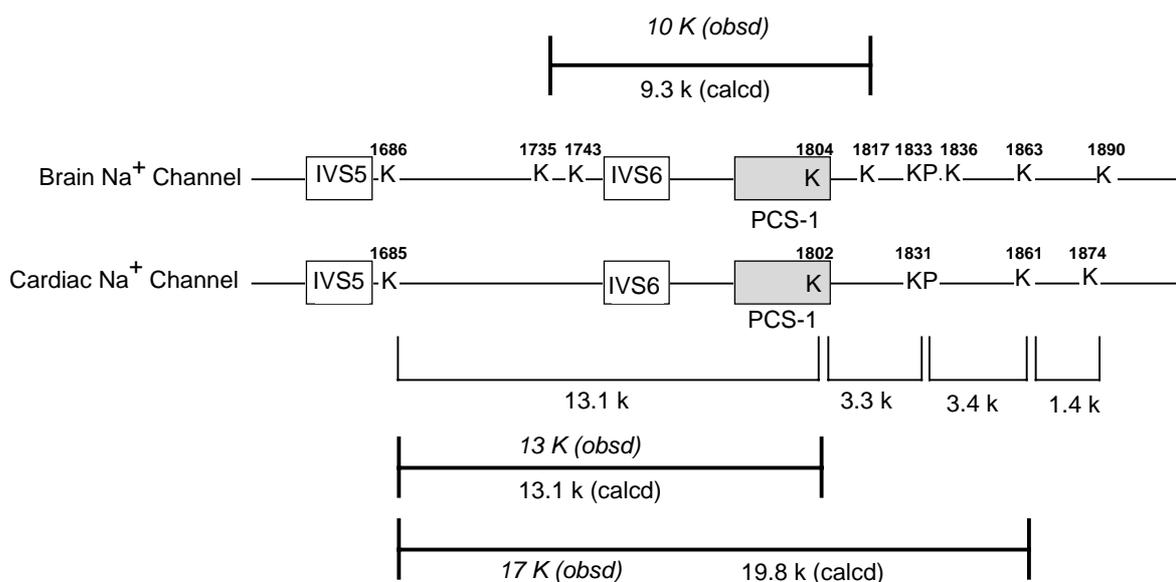


Figure 5. Location of photolabeled fragments in the IVS6 region of brain and cardiac Na⁺ channels.

Schematic alignments of the transmembrane segments S5 and S6 in domain IV and the antibody used (*shaded*). K and P mean Lys and Pro residues, respectively. Photolabeled peptide fragments observed as 10 kDa (brain Na⁺ channel) and 13- and 17- kDas (cardiac Na⁺ channel) and their calculated sizes are also indicated.

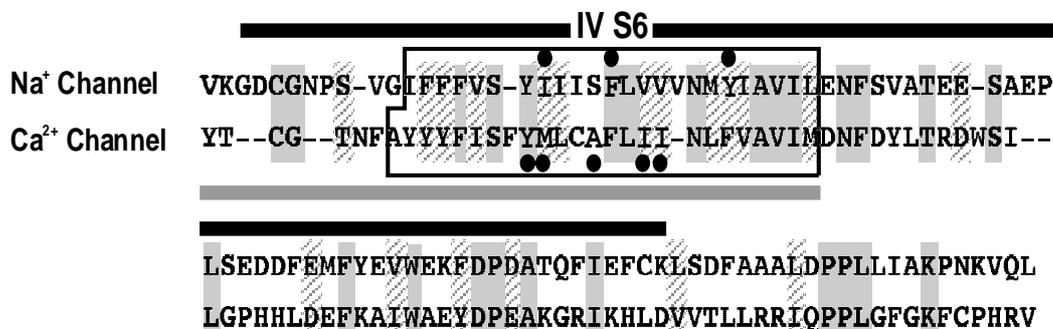


Figure 6. Comparison of primary structures that were identified as photolabeled regions in two Na⁺ channels and a Ca²⁺ channel. Boxes that are *shaded* and *hatched* indicate identical and similar amino acid, respectively. Bars that are *filled* and *shaded* indicate the peptide fragments photolabeled by (*R*)- and (*S*)- [³H]-D51-4700 in Ca²⁺- and Na⁺- channel, respectively. *Filled circles* are amino acid residues that are reported to be critical for drug bindings by photoaffinity labeling and site-directed mutagenesis.^{8-12, 28}

and/or domains also participate to form the binding sites, and (2) three-dimensional (3D) structure of this region is different between Na⁺ - and Ca²⁺ -channels, which may be expected partly by their different subunits-composition.^{19,20}

Initial works to reveal the 3D structures of Na⁺ channel²¹ and L-type Ca²⁺ channel²² have started, although their resolution is still low in single particle analysis by electron microscopic technique. We must wait, for a while, for the exact answer of the enantioselectivity until the 3D structures of the complex between each channel and corresponding enantiomer of semotiadil will be resolved.

3. Drug binding sites in Na⁺ - and Ca²⁺ -channels - Common or unique? It is worth noting that the identified binding region for (*S*)-semotiadil (a potential anti-arrhythmic agent) is largely overlapped with that for the etidocaine (Figure 6), a local anesthetic in brain Na⁺ channel.²⁸ Moreover, it is intriguing to have found in this study that the identified (*S*)-semotiadil binding region in Na⁺ channels have a highly homologous sequence to not only that for (*R*)-semotiadil but also those for typical Ca²⁺ anatagonists (DHP, PAA, and BTZ) previously reported in L-type Ca²⁺ channels.⁸⁻¹² Anti-arrhythmic agents for Na⁺ channels and Ca²⁺ anatagonists for L-type Ca²⁺ channels have a common feature to block the activity of their corresponding channels. It might explain partly that the common regions accommodate the binding sites for the drugs with different chemical structures.

However, the photolabeling and site-directed mutagenesis studies of L-type Ca²⁺ channels demonstrated that binding sites for the Ca²⁺ anatagonists are composed of IVS6 and other regions as well. Therefore, it is likely that (*S*)-semotiadil binding site in the Na⁺ channel is also composed of regions containing IVS6 and other segments/motifs as well. Such a factor may also play a role for Na⁺ - and Ca²⁺ -channels to discriminate each drug.

In summary, we identified, in this paper, that the (*S*)-semotiadil binding regions in brain and cardiac Na⁺ channels were similar to the (*R*)-semotiadil binding region in L-type Ca²⁺ channels, and these regions are not considered as a sole determinant for the enantioselectivity of semotiadil binding in Na⁺ - and Ca²⁺ - channels.

EXPERIMENTAL

Materials

To obtain (*S*)- and (*R*)-[³H]D51-4700, the corresponding precursors were synthesized by Prof. Honda's group similarly as described,²³ followed by radiolabeling with [³H]CH₃I in Tokai Laboratory of Daiichi Chemical Co., to yield the objective compounds with specific radioactivity of 2.9 and 2.8 TBq/mmol, respectively. Unradiolabeled (*S*)- and (*R*)-semotiadil were obtained from Daiichi Pharmaceutical Co., and flecanide was from Eisai Pharmaceutical Co. *Achromobacter lyticus protease I* (Lys-C) was purchased from Wako Pure Chemicals.

Peptide synthesis and antibody production

A polyclonal antibody (anti-PCS-1 antibody) was raised in rabbits against a synthetic peptide (Ser-Glu-

Asp-Asp-Phe-Glu-Met-Phe-Tyr-Glu-Val-Trp-Glu-Lys) corresponding to a region of 1791-1804 of the rat brain Na⁺ channel α -subunit sequence.²⁴ The peptide was synthesized by a peptide synthesizer, Pioneer (Perceptive), according to the Fmoc chemistry. Conjugation of the peptide to thyroglobulin and immunization were carried out similarly as described previously.⁶ The raised antibody was confirmed to react with porcine cardiac Na⁺ channel α -subunit, although its sequence (Ser-Glu-Asp-Asp-Phe-*Asp*-Met-Phe-Tyr-Glu-*Ile*-Trp-Glu-Lys) has two different amino acid residues (indicated as italic) from the rat brain counterpart.

Photoaffinity labeling (Preparative scale)

(1) Na⁺ channels from rat brain – Synaptosome fractions from rat brain were prepared as described.²⁵ The synaptosome fractions (50 mg/50 mL of 25 mM Tris-HCl, pH 7.4) was incubated with 100 nM (S)-[³H]-D51-4700 for 1 h at 30 °C in the dark, followed by for 30 min on ice. The mixture was transferred into a chilled glass Petri dish on ice as a layer with 5 mm depth, and irradiated for 20 min with a 100 W black light/blue lamp (Ultra-Violet Products Inc., San Gabriel, CA., USA) at a distance of 10 cm. After photolysis, the synaptosome was centrifuged (126,600 x g_{max}, 40 min) and the pellet was resuspended with 20 mM Hepes-Tris, pH 7.4 (7 mL). The suspension was solubilized and purified by the described method by Hartshorne *et al.*²⁶ The purified sample was first treated with 1% 2-mercaptoethanol and 1.5% SDS in 0.1 M Tris-HCl, pH 8.3, at 30 °C for 30 min, followed by 85 mM iodoacetamide for 30 min. The sample was further purified by gel permeation liquid chromatography as described.⁸

(2) Na⁺ channels from porcine hearts – Sarcolemmal membranes (SL membranes) were prepared as described previously,²⁷ and the SL membranes were solubilized and purified partially by DEAE-Sepahdex and WGA-Sepharose columns as described.¹⁸ The purified sample (0.8 mL) was incubated with 200 nM (S)-[³H]-D51-4700 for 1 h on ice in the dark. The mixture was transferred into a chilled 24-well plate on ice as a layer of 5 mm depth, and irradiated for 20 min with the 100 W black light/blue lamp as described above at a distance of 10 cm. After photolysis, the irradiated sample was dialyzed against 2 mM Tris-HCl, pH 7.4, at 4 °C, and concentrated 10-fold with an Amicon concentrator at 4 °C.

Lys-C digestion

(1) Brain Na⁺ channels – The photolabeled and purified α -subunit (7,000 dpm, 1 μ g) was dissolved into 70 μ L of 50 mM Tris-HCl, pH 9.0, containing 0.05% SDS and 0.01% Triton X-100 and digested with Lys-C (15 μ g/mL) at 37 °C for 12 h. The reaction was stopped by heating at 90 °C for 3 min, followed by addition of 0.1 mM phenylmethanesulfonyl fluoride and adjusting the pH between 7 and 8 with 0.2 N HCl.

(2) Cardiac Na⁺ channels – The photolabeled and concentrated sample (0.1 mL) containing 0.3% Triton X-100 and 0.06% phosphatidyl choline was digested with Lys-C (1 mg/mL) at 37 °C for 12 h. The digestion was stopped and treated similarly as described above in brain Na⁺ channels.

Immunoprecipitation

Immunobeads (anti-PCS-1-antibody-bound protein A Sepharose CL-4B) was prepared similarly as

described.⁶ Photolabeled and Lys-C-digested Na⁺ channels (50-100 μ L) was incubated with the immunobeads (50 μ L) in buffer A (25 mM Tris-HCl, pH 7.2, 140 mM NaCl, and 0.1% Triton X-100) for 12 h at 4 °C. After washing the beads with buffer A, bound peptide fragments were eluted with either 0.1 M glycine-HCl, pH 3.0 or a sampling buffer (10 mM Tris-HCl, pH 7.6, 1% SDS, 20 mM dithiothreitol, 4 mM ethylenediaminetetraacetic acid, and 2% sucrose) for SDS-PAGE, in order to determine immunoprecipitated radioactivity or analyze the immunoprecipitated peptide fragments on SDS-PAGE, respectively.

SDS-PAGE and radioluminography

Photolabeled Na⁺ channels and their Lys-C digests were analyzed on SDS-PAGE using 6% and 15% polyacrylamide gel, respectively, according to Laemmli.²⁹ After electrophoresis, proteins and peptides in the gel were transferred onto polyvinylidene difluoride membrane and the membrane was contacted with an imaging plate, BAS-TR2040S (Fuji Photo Film Co.). The imaging plate was analyzed by a Bio-Imaging Analyzer BAS 1000 model, as described.⁶

Photoaffinity labeling (Competitive experiments)

Synaptosome fractions (2 mg/mL) from rat brain (for Na⁺ channels) were incubated with either (*S*)- or (*R*)-[³H]-D51-4700 (100 nM) in the presence or absence of excess amount (300-fold) of (*S*)- and (*R*)-semotiadil at 30 °C for 1 h and irradiated with the 100 W black light/blue lamp similarly as described above. Triad membranes (2 mg/mL) prepared as described⁶ from rabbit skeletal muscle (for L-type Ca²⁺ channels), were similarly incubated and irradiated. The photolabeled samples were solubilized in the sample buffer for SDS-PAGE and analyzed on SDS-PAGE and by radioluminography as described above. To determine the effect of flecanide ($K_i = 5.7 \mu\text{M}^{30}$) on photolabeling of Na⁺ channel with (*S*)-[³H]-D51-4700, 0.5 mM flecanide was used.

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REFERENCES

1. N. Miyawaki, F. Yamazaki, T. Furuta, T. Shigei, and H. Yamauchi, *Drug. Dev. Res.*, 1991, **22**, 293.
2. I. Kodama, R. Suzuki, K. Maruyama, and J. Toyama, *Br. J. Pharmacol.*, 1995, **114**, 503.
3. A. Hirasawa, A. Haruno, T. Matsuzaki, and K. Hashimoto, *Jpn. Heart J.*, 1992, **33**, 851.
4. S. Nagashima, T. Uematsu, T. Araki, M. Matsuzaki, H. Fukuchi, and M. Nakashima, *Naunyn-Schmied. Arch. Pharmacol.*, 1992, **345**, 688.
5. Y. Hatanaka, H. Nakayama, and Y. Kanaoka, *Rev. Heteroatom Chem.*, 1996, **14**, 213.

6. A. Kuniyasu, K. Itagaki, T. Shibano, M. Iino, G. Kraft, A. Schwartz, and H. Nakayama, *J. Biol. Chem.*, 1998, **273**, 4635.
7. N. Ii, A. Kuniyasu, K. Kawahara, T. Shibano, A. Schwartz, and H. Nakayama, *FEBS Lett.*, 1998, **441**, 83.
8. H. Nakayama, M. Taki, J. Striessnig, H. Glossmann, W. A. Catterall, and Y. Kanaoka, *Proc. Natl. Acad. Sci. USA.*, 1991, **88**, 9203.
9. J. Striessnig, H. Glossmann, and W.A.Catterall, *Proc. Natl. Acad. Sci. USA.*, 1990, **87**, 9108.
10. W.A. Catterall and J. Striessnig, *Trends Pharmacol. Sci.*, 1992, **13**, 256.
11. G. Varadi, Y. Mori, G. Mikala, and A. Schwartz, *Trends Pharmacol. Sci.*, 1995, **16**, 43.
12. W. A. Catterall, *Ann. Rev. Cell Dev. Biol.*, 2000, **16**, 521.
13. H. Glossmann and J. Striessnig, *Rev. Physiol. Biochem. Pharmacol.*, 1990, **114**, 1.
14. H. Nakayama and Y. Kanaoka, *Heterocycles*, 1996, **42**, 901.
15. H. K. Motoike, I. Bodi, H. Nakayama, A. Schwartz, and G. Varadi, *J. Biol. Chem.*, 1999, **274**, 9409.
16. M. Noda, S. Shimizu, T. Tanabe, T. Takai, T. Kayano, T. Ikeda, H. Takahashi, H. Nakayama, Y. Kanaoka, N. Minamino, K. Kangawa, H. Matsuo, M. A. Raftery, T. Hirose, S. Inayama, H. Hayashida, T. Miyata, and S. Numa, *Nature*, 1982, **312**, 121.
17. H. Nakayama, Y. Hatanaka, E. Yoshida, K. Oka, M. Takanohashi, Y. Amano, and Y. Kanaoka, *Biochem. Biophys. Res. Commun.*, 1992, **184**, 900.
18. A. Lombet and M. Lazdunski, *Eur. J. Biochem.*, 1984, **141**, 651.
19. A. L. Goldin, *Curr. Opin. Neurobiol.*, 1993, **3**, 272.
20. W. A. Catterall, *Science*, 1991, **253**, 1499.
21. C. Sato, Y. Ueno, K. Asai, K. Takahashi, M. Sato, A. Engel, and Y. Fujiyoshi, *Nature*, 2001, **409**, 1047.
22. K. Murata, N Odahara, A. Kuniyasu, Y. Sato, H. Nakayama, and K. Nagayama, *Biochem. Biophys. Res. Commun.*, 2001, **282**, 284.
23. Y. Watanabe, K. Osanai, T. Nishi, N. Miyawaki, D. Shii, T. Honda, and T. Shibano, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 1923.
24. M. Noda T. Ikeda, T. Kayano, H. Suzuki, H. Takeshima, M. Kurasaki, H. Takahashi, and S. Numa, *Nature*, 1986, **320**, 188.
25. T. Ueda, P. Greengard, Berzins, R. S. Cohen, F. Blomberg, D. J. Grab, and P. Siekevitz, *J. Cell Biol.*, 1979, **83**, 308.
26. R. P. Hartshone and W. A. Catterall, *Proc. Natl. Acad. Sci. USA.*, 1981, **78**, 4620.
27. A. Kuniyasu, K. Oka, T. Ide-Yamada, Y. Hatanaka, T. Abe, H. Nakayama, and Y. Kanaoka, *J. Biochem.*, 1992, **112**, 235.
28. D. S. Ragsdale, J. C. Mcphee, T. Scheuer, and W. A. Catterall, *Science*, 1994, **265**, 1724.
29. U. T. Laemmli, *Nature*, 1970, **227**, 680.
30. W. Voigt and R. Mannhold, *Eur. J. Pharmacol.*, 1989, **169**, 103.