Photoaffinity Labeling of the Electroplax Sodium Channel with Tetrodotoxin Derivatives. II.[1] Comparison of the Photoreactivity of Different Photoactivable Groups in the Tetrodotoxin Binding Site[2]

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Four photoactivable tetrodotoxin (TTX) derivatives and the corresponding tritiated compounds were synthesized and purified. The photoactivable groups introduced were a nitro-azidophenyl (NAP) group and a trifluoromethyl diazirinobenzoyl (TDB) group, as nitrene and carbene precursors, respectively, as well as two isomers of the fluoro-nitrophenoxy group, a new photoreactive group selective for nucleophiles. The binding affinities of the four derivatives to the sodium channel were similar to that of TTX, but the values of specific photoincorporation were unexpectedly low (up to 2.5%), suggesting that the photoactivable groups are likely to be oriented unfavorably for labeling reactions in the TTX binding site. Two of the labeled sodium channel proteins were purified and digested. Peptides labeled with TTXenNAP lost most of the label during high performance liquid chromatography in 0.1% trifluoroacetic acid–acetonitrile, while peptides labeled with TTXenTDB retained the labels during the procedures. The TTXenTDB-labeled peptides were eluted in four major fractions with 80% recovery of the amount applied on a reverse-phased C4 column. However, further purification is necessary to identify the labeled sites of the peptides.

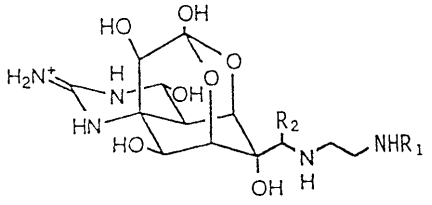
Keywords photoaffinity labeling; tetrodotoxin derivative; nitrene; carbene; photoactivated aromatic; eel sodium channel; binding; reactivity

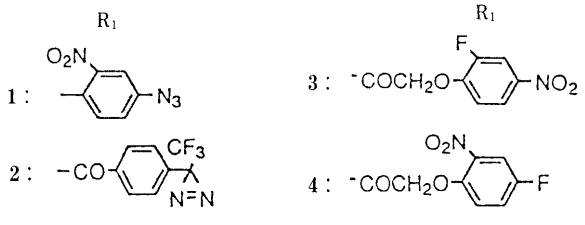
Voltage-sensitive sodium channels are the molecular structures responsible for the increase in sodium permeability during the initial phase of the action potential in the most excitable cells, such as nerve, muscle and heart cells. Since the complete amino acid sequence of the sodium channel from eel electroplax was first elucidated,[3] followed by those from rat brain,[4] *Drosophila mercatorum*,[5] and rabbit skeletal muscle,[6] more defined approaches at the molecular level have become feasible to investigate the structural features which are essential to express the molecular functions. Five distinct groups of neurotoxins which specifically bind to the sodium channels and modify their functional properties[7] are useful molecular probes to reveal the molecular architectures of the ion channels relevant to their functions. Tetrodotoxin (TTX) is a typical sodium channel toxin, which binds to the channel with high affinity and blocks the passage of sodium ions across the membrane.

Photoaffinity labeling has proved to be a powerful tool in the identification of the polypeptide components of receptors and ion channels, which are not expected to contain particularly reactive groups in the way that enzyme active sites do. The neurotoxins specific for the sodium channel provide potentially useful reagents for the preparation of probes for photoaffinity labeling. We have already demonstrated a specific photolabeling with a TTX derivative.[1] As a continuation of this work, we synthesized three new TTX derivatives with different photoactivable groups and applied them to the photoaffinity labeling of the sodium channel from electric eel electroplax. In this paper we describe the synthesis and binding activity of the photoactivable TTX derivatives, and the results of photolabeling are compared in detail. We also describe attempt to separate the labeled peptide fragments by high performance liquid chromatography (HPLC).

Results and Discussion

Synthesis TTX was first oxidized by means of the Moffatt reaction and then photoactivable groups were introduced *via* ethylenediamine as a spacer molecule, by reductive alkylation with $NaBH_3CN$, or $NaB[^3H]_3CN$ for the corresponding radiolabeled compounds (Chart 1). This method was initially reported by Balerna *et al.*[8] to prepare TTXen nitro-azidophenyl (NAP), but we modified the reaction conditions and purification procedures. Oxidation was carried out under the conditions described by Bontemps *et al.*[9] except that a 14-fold higher concentration of TTX (0.3 μM) was used and in the coupling step 2 eq of 2-nitro-4-azidophenylethylenediamine (NAPen) with respect to initial TTX was used. HPLC analysis of the conventionally purified TTXenNAP[8] revealed that it still contained some NAPen, as shown in Fig. 1. After purification, the overall yield of TTXenNAP was 6%[1] which was very low but still much higher than the reported value of 1—2%.[8] Other TTX derivatives and the tritiated derivatives were similarly synthesized and purified except for slight variations in the amounts of ethylenediamine and $NaBH_3CN$ amounts used. The results are summarized in Table I.





R_2= H or [^{3}H]

Chart 1

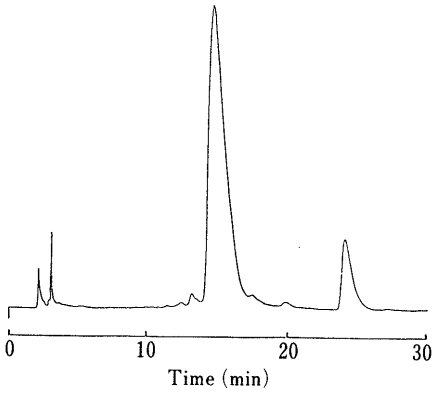


Fig. 1. HPLC Profile of TTXenNAP after Conventional Purification on a Cellulose Thin-Layer Plate

Two-dimensionally separated and extracted[8] material was loaded on a Nucleosil 5C18 column (4.6×250 mm) and developed with aqueous trifluoroacetic acid (pH 2.6) containing 15% (v/v) acetonitrile at a flow rate of 1.0 ml/min. TTXenNAP was eluted at 15 min and NAPen at 24.5 min.

TABLE I. Amounts of Reagents Used to Form Some Photoactivable TTX Derivatives and Yields of the Products

Compound	TTX (μmol)	Ethylene-diamine derivative (μmol)	$NaBH_3CN$ or $NaB[^3H]_3CN$ (μmol)	TTX derivative formed (μmol)	Yield (%)
TTXenNAP	3.14	3.15	1.0	0.198	6.3
TTX[^{3}H]enNAP	6.28	3.0	2.0	0.109	1.7
TTXenTDB	6.28	3.0	6.3	0.062	1.0
TTX[^{3}H]enTDB	15.7	7.8	4.0	0.148	1.0
TTXen2FNP	3.14	1.6	3.1	0.014	0.5
TTX[^{3}H]en2FNP	3.14	1.6	1.0	0.023	0.7
TTXen4FNP	3.14	1.6	3.1	0.015	0.5
TTX[^{3}H]enFNP	3.14	1.6	1.0	0.022	0.7

Reducing the amounts of ethylenediamine derivatives from equimolar to TTX to 0.5 eq mol lowered the overall yields almost proportionally, while decrease in $NaBH_3CN$ had no significant effect, which suggests that the amount of a Schiff base formed from the oxidized TTX and the ethylenediamine derivatives is critical for the overall reactions and its amount is not so high as the minimum molar fraction of $NaBH_3CN$ (0.3 eq with respect to TTX) applied here.

It was difficult to obtain enough of the purified TTX derivatives for elemental analysis or nuclear magnetic resonance (NMR) spectroscopy, because the overall yields were quite low as described. Fast atom bombardment (FAB) mass spectroscopic analysis did not show any significant peaks. However, the four HPLC-purified derivatives showed characteristic absorption spectra which correspond to those of the photoactivable chromophore groups. Their binding characteristics (described below) also support the view that they are TTX derivatives. The position of the TTX molecule at which the photoactivable groups are introduced remains undetermined, but C-11 is the most probable position, as described,[11] because it is the only primary alcohol in the TTX molecule and chemical modification of all other hydroxyls has been reported to decrease the biological activities of TTX.[11-13]

Binding Activity Binding activity of the synthesized TTX derivatives to the solubilized electroplax sodium channel was measured in two ways. The first was assay of competitive binding to the sodium channel with respect to tritiated TTX–Lys. As shown in Fig. 2, all the TTX derivatives, **1**, **2**, **3**, and **4**, inhibited the TTX–[^{3}H]Lys binding as effectively as TTX did, and their IC_{50} values were 60, 70, 55, and 85 nM, respectively, which were only 2.1-fold larger, at most, than that of TTX, 40 nM, under the conditions used. The second method was direct binding assay of the tritiated photoactivable TTX derivatives to the sodium channel. Figure 3 shows the results of TTX[^{3}H]en trifluoromethyl diazirinobenzoyl (TDB) binding, and its specific binding component after subtracting the nonspecific binding fractions which were measured in the presence of TTX. By analyzing the Scatchard plot of the specific binding component (depicted in Fig. 3 insert), K_d

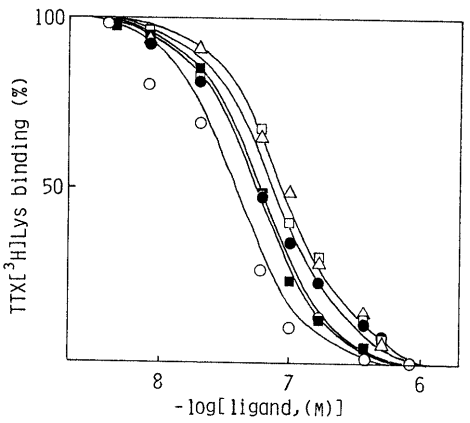


Fig. 2. Inhibition Dose–Response of the Photoactivable TTX Derivatives against TTX[^{3}H]Lys Binding to the Electroplax Membrane Fragments

Increasing concentrations of each of the derivatives (■, TTXenNAP; □, TTXenTDB; ●, TTXen2FNP; △, TTXen4FNP; and ○, TTX) were incubated in the dark with 20 nM TTX[^{3}H]Lys and the membrane fragments (4 mg protein/ml) in 0.5 ml of potassium phosphate buffer (pH 7.5) at 0 °C for 30 min. After centrifugation at 1000×*g* for 15 min, 200 μl aliquots of the supernatant were counted for radioactivity.

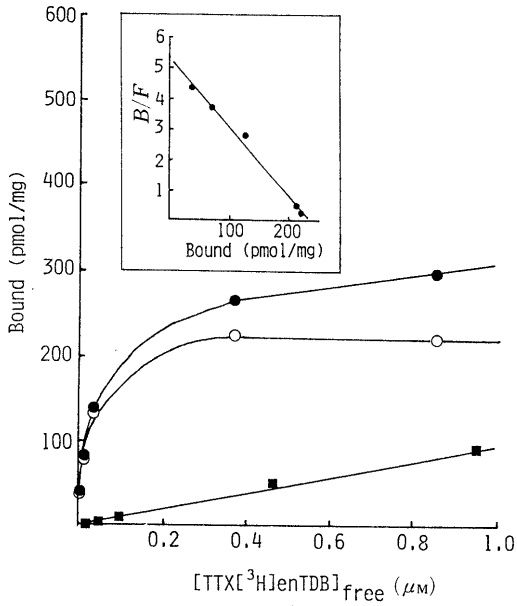


Fig. 3. Binding of TTX[^{3}H]enTDB on Solubilized and DEAE-Sephadex-Purified Electroplax Sodium Channel by Rapid Filtration Assay

Data represent bound, *B* (pmol/mg protein), *versus* free, *F*(μM), toxin concentration in the absence (●) and presence (■) of an excess of unlabeled TTX. Specific binding, obtained by subtracting *B* in the presence of TTX from *B* in the absence of TTX, is also shown (○). The insert depicts a Scatchard plot for the specific binding: *B*/*F* (pmol/mg protein/nM) *versus* *B* (pmol/mg protein). Binding parameters: $K_d = 45$ nM and $B_{max} = 230$ pmol/mg protein.

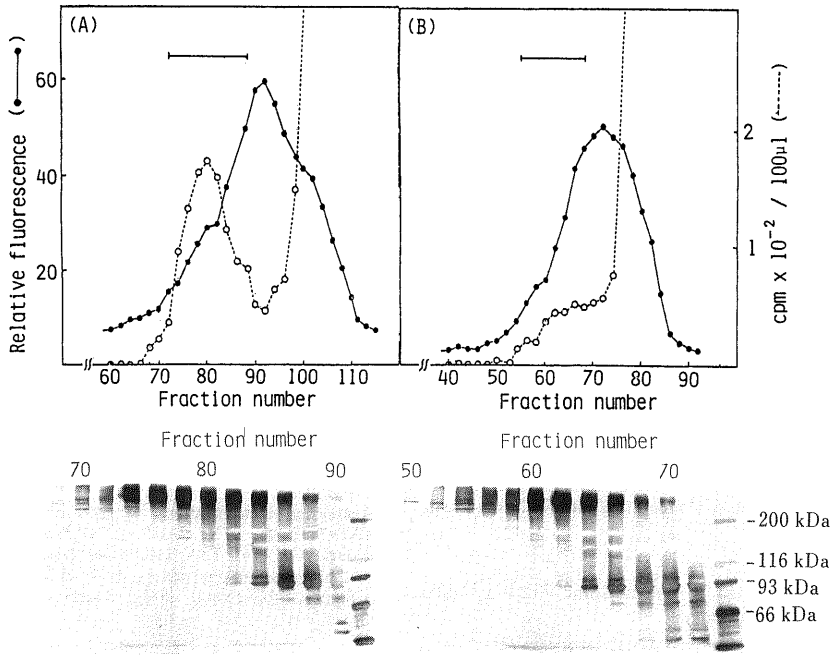


Fig. 4. Sepharose 4B-Cl Chromatography of the Photolabeled Sodium Channel with TTX[^{3}H]enTDB and SDS-PAGE of the Eluted Fractions

(A) Photolabeled sample was heat-denatured, subjected to reductive carboxymethylation, and developed on a column (3.2 × 85 cm) with 50 mM sodium phosphate buffer (pH 7.5) containing 0.1% SDS at a flow rate of 16 ml/h. Fraction of 5.5 ml were collected. Protein and radioactivity were monitored by fluorescamine assay and liquid scintillation counting, respectively. Polypeptide compositions of the fractions 70—90 were analyzed by SDS-PAGE on 6% polyacrylamide gel and silver-staining (lower panel). Fractions 72—88 were pooled for subsequent use. (B) A sample photolabeled in the presence of unlabeled TTX was similarly developed on a column (1.5 × 85 cm) at a flow rate of 10 ml/h (2 ml/fraction). Fractions were similarly monitored and fractions 55—68 were pooled.

Table II. Comparison of Amounts of Synthesized TTX Derivatives Photoincorporated into the Electroplax Sodium Channel

Ligand	Incorporation without TTX (pmol)	Incorporation with TTX (pmol)	Specific incorporation (pmol)	Specific incorporation (pmol/mg)	Specific incorporation (%)
TTX[^{3}H]enNAP	76	55	21	1.9	0.2
TTX[^{3}H]enTDB	325	52	273	25.2	2.5
TTX[^{3}H]en2FNP	64	57	7	0.6	0.06
TTX[^{3}H]en4FNP	66	64	2	0.2	0.02

and B_{max} values were determined as 45 nM and 230 pmol/mg, respectively. Other derivatives were analyzed similarly and the values of K_d and B_{max} obtained for the tritiated TTX derivatives of NAP and 2-fluoronitrophenol (2FNP), were 53 nM and 275 pmol/mg and 41 nM and 203 pmol/mg, respectively. The B_{max} values of all the TTX derivatives were similar, and their K_d values were 45—60 nM: only a 1.5- to 2-fold decrease in affinity was observed when compared with that of TTX (30 nM).[14]

All the photoactivable TTX derivatives showed very little difference from TTX in their binding affinity and maximal binding, demonstrating that there is essentially no interference with their binding to the TTX binding site after introduction of the photoactivable groups onto the TTX molecule.

Photolabeling and Its Efficiency Solubilized and partially purified sodium channel (11 nmol and 1 μM of TTX[^{3}H]Lys binding sites) was incubated with each of the photoactivable TTX derivatives (1 μM) and irradiated. Under the equilibrated incubation conditions used here 80% of the sodium channel used was observed to form a complex with the reagent. The irradiated sample was heat-denatured with 2-mercaptoethanol/sodium dodecyl sulfate (SDS) followed by carboxymethylation, and then purified by gel-filtration chromatography on Sepharose 4B. Eluted sodium channel fractions of high molecular weight (*ca.* 250 kilodaltons (kDa))[15,16] monitored by SDS-polyacrylamide gel electrophoresis (PAGE), were pooled (Fig. 4) and the photolabeled amount of radioactivity/protein molecule (Table II) was determined. Amounts of nonspecific photolabeling were determined to be 52—64 pmol per 11 nmol of the sodium channel, namely 0.5—0.6%, being virtually independent of the nature of the photoactivable groups introduced. The specifically photolabeled amounts, however, varied with the photoreactive groups: 2.5% for the diazirine compound, 0.2% for the azido compound, and less than 0.1% for the fluoro-nitroanisoles. The two fluoro-nitroanisoles, new photoactivable groups that react selectively with nucleophiles,[17] gave essentially no photolabeling. The results indicate that there are very few nucleophilic groups of the channel protein available to react in the region accessible to the photoreactive fluoro-nitroanisoles. The phenyldiazirine derivatives showed more than 10-fold greater photolabeling efficiency than the nitrophenylazide. This is interpreted in terms of higher reactivity of the phenylcarbene, the reactive species photogenerated from the phenyldiazirine, than that of the nitrophenylnitrene produced from the nitrophenylazide; the life time of the phenylcarbene (0.1—1 μs[18]) is much shorter than that of the nitrophenylnitrene (*ca.* 10 μs[19]).

The specifically incorporated amounts varied depending on the photoactivable groups employed, but their efficiency was quite low, up to 2.5% with the phenyldiazirine, even under conditions where 80% of the applied sodium channel formed the complex with the photoactivable at reagent. The observed low efficiency in photolabeling, although all the TTX derivatives synthesized here have similar binding

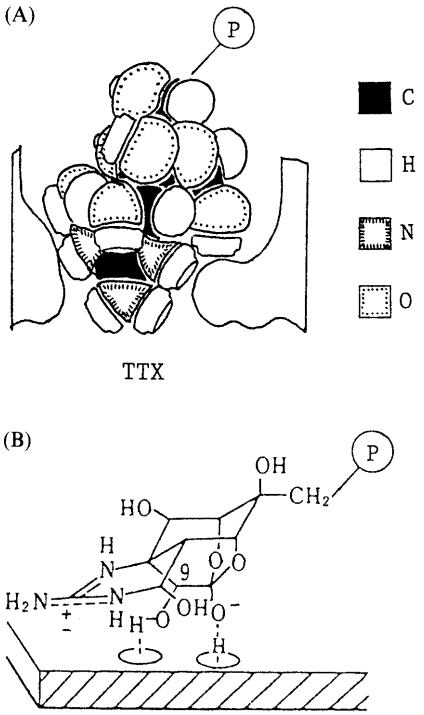


Fig. 5. Probable Binding Modes of the Synthesized Photoactivable TTX Derivatives in the TTX Binding Site of the Sodium Channel According to Two Proposed Models

In these models, (A) the "plugging" model[20] or (B) the "three-point binding" model,[21] photoactivable groups (indicated as P), which are assumed to be introduced onto C-11 of TTX, may orient away from the guanidinium group (model A) or lie a little far from the channel surface (model B).

affinity to TTX, suggests that the photoactivable groups are likely to be located unfavorably for their reactions in the TTX binding loci in the sodium channel. Two models have been proposed for TTX binding to the sodium channels, namely the classical "plugging" model[20] and the "three-point binding" model.[21] In Fig. 5, probable binding modes of the photoactivable TTX derivatives to the sodium channels are illustrated, according to the two proposed models and assuming that the photoactivable groups are introduced onto the C-11 position of TTX. Although the position where the photoactivable groups were introduced in the TTX molecule was not well defined chemically as described above, the present findings of low efficiency in incorporation may be consistent with introduction of the photoreactive groups at the C-11 position of TTX.

Nature of the Peptide Fragments Photolabeled with TTXenNAP and TTXenTDB In order to separate the photolabeled peptide(s) of sodium channel protein, enzyme digestion followed by HPLC separation was carried out for the two proteins which were labeled with TTXenNAP and TTXenTDB.

First the purified sodium channels which were photolabeled with TTXenNAP in the presence or absence of a large excess of TTX were digested with lysyl endo-protease and the digests were subjected to HPLC separation on a C18 reversed-phase column. By comparing the two chromatograms shown in Fig. 6, specifically photolabeled peptides were observed to be eluted mainly at 33—34 min, and also around 36 and 52 min to some extent. To purify the major 33—34 min peak further, the pooled fractions were evaporated *in vacuo*, resuspended in 0.1% trifluoroacetic acid and rechromatographed with a solvent system of

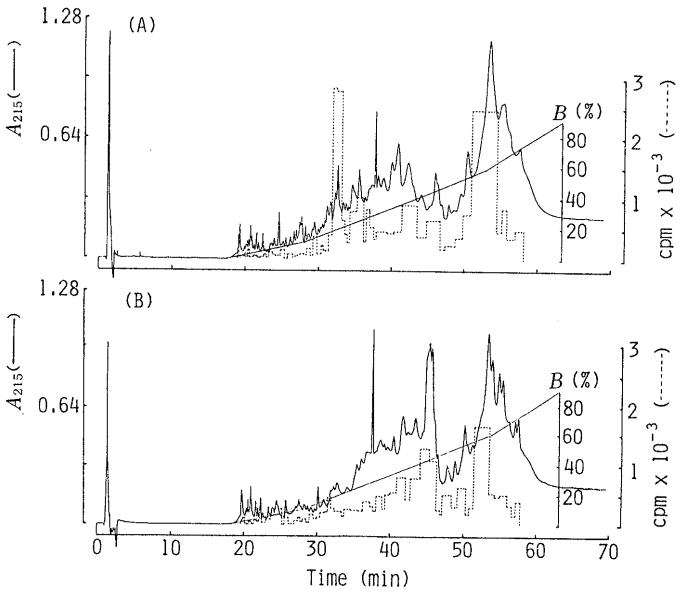


Fig. 6. Fractionation of Lysyl Endoprotease-Digested Peptides from TTX[^{3}H]enNAP-Labeled Sodium Channel by Reverse-Phase HPLC

(A) Enzyme digest from the photolabeled sodium channel was applied to a C18 column equilibrated in 0.1% trifluoroacetic acid elution was carried out at 1.0 ml/min with a gradient of solvent B, acetonitrile–2-propanol (3:7, v/v), as indicated. The eluate was monitored by measuring the absorbance at 215 nm (solid line), and radioactivity contained in fractions (broken line) collected at 1 min intervals was measured by subjecting aliquots (50 μl) to liquid scintillation counting. (B) An elution profile of the photolabeled sodium channel in the presence of unlabeled TTX followed by parallel treatment.

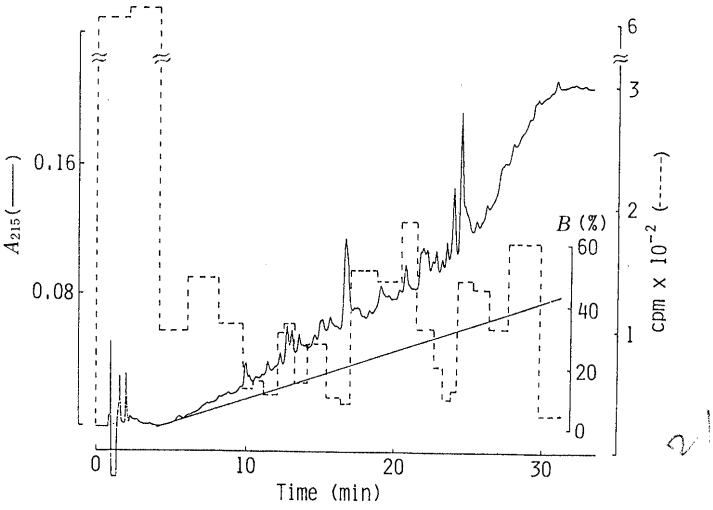


Fig. 7. Rechromatography of the Pooled Fraction (Eluted at 33—34 min in Fig. 6) by Reverse-Phase HPLC.

The pooled fraction was loaded on a C18 column equilibrated in 10 mM ammonium acetate (pH 6.0) containing 5% acetonitrile, and eluted at 1.0 ml/min with a gradient of solvent B, 10 mM ammonium acetate (pH 6.0) containing 60% acetonitrile, as indicated. Monitoring of the eluate was carried out as described in the legend to Fig. 6.

10 mM ammonium acetate (pH 6.0)/acetonitrile (Fig. 7). The majority of the radioisotope was recovered at the front, where not peptides appeared. This observation suggests that the photoproducts of TTXenNAP with the peptide(s) were quite unstable under the conditions used. Such chemical lability of nitrene-derived photoproducts has been reported recently.[22-24] Although the chemical basis of the instability is not well documented yet, it is a major obstacle to identification of the photolabeled sites.

Secondly, the TTXenTDB-labeled sodium channel was digested with trypsin followed by HPLC separation on a C4

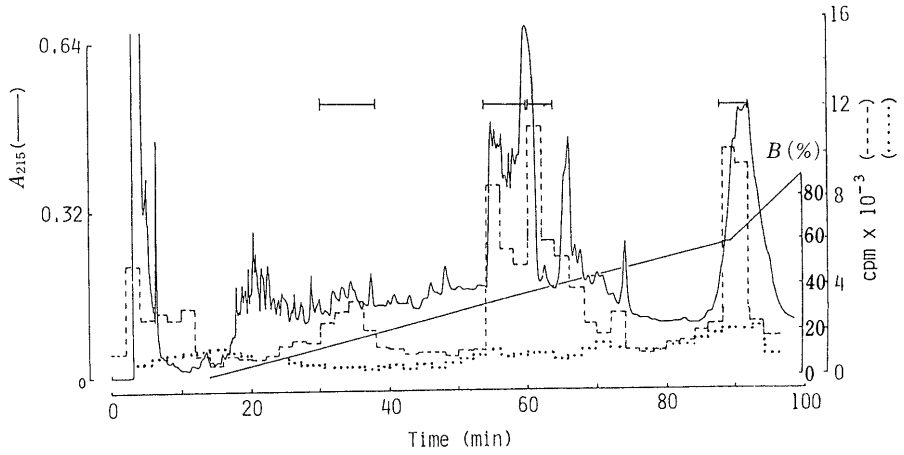


Fig. 8. Fractionation of Tryptic Peptides from TTX[^{3}H]enTDB-Labeled Sodium Channel by Reverse-Phase HPLC

The enzyme digest was applied to a C4 column equilibrated in 0.1% trifluoroacetic acid and developed at 0.5 ml/min with a gradient of solvent B, acetonitrile–2-propanol (7 : 3, v/v), as indicated. Monitoring of the eluate was carried out by measuring the absorbance at 215 nm and radioactivity measurement as described in the legend to Fig. 6. Parallel treatment of the sodium channel photolabeled in the presence of unlabeled TTX produced a similar A_{215} profile (not shown) and the radioactivity profile shown here (dotted line).

reverse-phase column in this case, because only 25% of the applied radioisotope was recovered from the digest of TTXenNAP-labeled channel on a C18 column. The elution profile is shown in Fig. 8; the isotope recovery increased to 80% in this system.

As no siginificant radioisotope peak was observed for the photolabeled sample in the presence of excess TTX, the eluted radioisotope peaks can be considered as specifically photolabeled peptides. However, they were apparently eluted as at least four groups. The fact that the number of labeled peptides was more than expected, again suggested that the photoactivable TDB group may be located rather loosely near the TTX binding site. Rechromatographic trials to purify these labeled peptides have so far been unsuccessful. Studies along this line are currently under way, with a view to accumulating enough material to allow identification of the labeled sites.

Experimental

Synthesis **1. TTXenNAP** NAPen was prepared by a modification of the procedure of Chicheportiche *et al.*,[25] where mono-*N*-(*tert*-butyloxycarbonyl)ethylenediamine instead of ethylenediamine was reacted with 2-nitro-4-azidobenzene fluoride in CH_2Cl_2 for 24 h at room temperature. The product was purified by Al_2O_3 column chromatography ($CHCl_3$: MeOH = 4 : 1) followed by treatment with 2 N HCl/ethyl acetate. Orange needles were obtained in 89% yield after recrystallization from ether/*n*-hexane, mp 64—66 °C (lit. 64—68 °C).[26] NAPen was then coupled to TTX by following a method similar to that described by Balerna *et al.*,[8] but with some modifications. Typically, Moffatt oxidation of TTX (1 mg, 3.14 μmol) was carried out in 20 μl of dry dimethylsulfoxide (DMSO), a 14-fold higher concentration of TTX (0.3 μM) than that employed by Bontemps *et al.*,[9] and equimolar of NAPen with respect to initial TTX was used in the coupling step. The reaction mixture was separated on a cellulose plate as described,[8] but the separated and extracted material (TTXenNAP) was still contaminated with NAPen as determined by HPLC analysis. HPLC conditions were as follows: column, Nucleosil 5C18 (4.6 × 250 mm, Nagel); solvent, 15% acetonitrile in aqueous trifluoroacetic acid solution (pH 2.6); flow rate, 1.0 ml/min; detection wavelength, 260 nm. The purified TTXenNAP, which was eluted at 14 min, was pooled and the solvent was removed by evaporation. The residue was dissolved in 1 mM acetic acid (1 ml) and the yield was determined as 6.3% from the absorbance at 462 nm due to the NAPen moiety ($\varepsilon = 4700$).

Tritiated TTXenNAP was similarly prepared except that 0.3 eq of NaB[^{3}H]$_3$CN (20 mCi, Amersham) and 0.5 eq mol of NAPen with respect to TTX (6.28 μmol) were used and the reaction mixture was directly purified by HPLC under the same conditions. The amount of the purified compound was determined as 109 nmol (1.7% yield) from its radioactivity and absorbance at 462 nm, and its specific radioactivity was 4.5 Ci/mmol.

2. TTXenTDB TDB acid was synthesized in seven steps from *p*-bromobenzoic acid *via* 2-{*p*-(trifluoroacetyl)phenyl}-4,4-dimethyl-2-oxazoline; the procedures will be described in detail elsewhere. TDBen was prepared by coupling the *N*-hydroxysuccinimide ester of TDB acid with *N*-mono(*tert*-butyloxycarbonyl)ethylenediamine followed by treatment with 2 N HCl/ethyl acetate. TTXenTDB was synthesized similarly to TTXenNAP. Briefly, lyophilized powder of the trifluoroacetic acid salt of TTX (starting from 2 mg, 6.28 μmol of citrate-free TTX obtained from Sankyo Chemical Co., Tokyo) was dried over P_2O_5 *in vacuo*. The dried powder was dissolved in 21 μl of DMSO, 3.2 μl of 1 M H_3PO_4 (3.2 μmol) in dry DMSO and 15.8 μl of 2 M dicyclohexylcarbodiimide (31.6 μmol) in dry DMSO were added and the whole was stirred for 5 h at 40 °C. The resultant suspension was treated as described,[10] and the aqueous phase (50 μl) containing oxidized TTX was mixed with TDBen (0.8 mg, 3 μmol) in 100 μl of methanol and stirred at room temperature for 3 h. The pH of the reaction mixture was adjusted to 6.0 with acetic acid, then 63 μl of 0.1 M $NaBH_3CN$ (6.3 μmol) was added and the reaction was allowed to proceed at 40 °C for 5 h. The reaction mixture was directly subjected to HPLC purification under the following conditions: column, Nucleosil 5C18 (4.6 × 250 mm); solvent, 20% acetonitrile in aqueous trifluoroacetic acid (pH 2.6); flow rate, 0.8 ml/min; and detection wavelength, 348 nm. TTXenTNB was eluted at 14.4 min (62 nmol, 1%). Tritiated TTXenTDB was similarly synthesized by starting with 5 mg (15.7 μmol) of TTX, but 4 μmol of NaB[^{3}H]$_3$CN (50 mCi) was used to yield 148 nmol of the desired product with a specific radioactivity of 4.0 Ci/mmol.

3. TTXen2FNP and TTXen4FNP These two compounds were synthesized similarly to TTXenTDB except that *N*-mono(3-fluoro-4-nitrophenoxy)acetyl ethylenediamine (2FNPen) or *N*-mono(4-fluoro-2-nitrophenoxy)acetylethylenediamine (4-FNPen) was used instead of TDBen. The amounts of the products obtained were determined by measuring the absorbance at 314 nm (based on the value of $\varepsilon = 10120$ for 2-fluoro-4-nitroanisole) or at 314 nm ($\varepsilon = 2880$ for 4-fluoro-2-nitroanisole), respectively, as well as the radioactivities of the corresponding tritiated compounds (see Table I). Specific radioactivity obtained was 1.2 Ci/mmol for both compounds. 2FNPen and 4FNPen were derived from the corresponding fluoronitrophenoxyacetic acids and *N*-mono(*tert*-butyloxycarbonyl)ethylenediamine followed by 2 N HCl/ethyl acetate treatment. Two fluoronitrophenoxyacetic acids were prepared from fluoronitrophenols as starting materials. The synthesis and properties of these compounds will be described elsewhere.

Binding Studies **1. Competetive Binding Assays** Tritiated TTX–Lys was prepared by the reported method as described.[10] Mixture of 20 nM TTX[^{3}H]Lys and the membrane fragments of electric eel electroplax (prepared according to the method described[27]) were incubated with various concentrations of either of the synthesized photoactivable TTX derivatives in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.5) at 0 °C in the dark. After 30 min, the mixture centrifuged in a microfuge (Kubota KR-180A) at 10000 × ***g*** for 15 min and the radioactivities of 200 μl aliquots of the supernatant (duplicate) were counted.

2. Saturation Assays Increasing concentrations of the tritiated photo-

activable TTX derivatives (20—1000 nM) were incubated in the dark with solubilized and diethylaminoethyl (DEAE)-Sepahdex purified electroplax sodium channels[15] (50 μg of protein) in 0.2 ml of 50 mM potassium phosphate buffer (pH 7.5) at 0 °C for 30 min and 180 μl aliquots were rapidly filtered through a small column of Sephadex G-50 as described.[28] Nonspecific binding was measured in the presence of an excess of unlabeled TTX at a 40 μM concentration. Specific binding was obtained by subtracting the nonspecific binding from the total binding and was analyzed by means of the Scatchard plot.

3. Photolabeling Experiments Solubilized and DEAE-Sephadex purified sodium channel (11 nmol of TTX[^{3}H]Lys binding sites) in 10 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 0.3% Lubrol PX/phosphatidylcholine (molar ratio of 7:1) and four protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM iodoacetamide, 1 μM pepstatin A, and 5 mM ethylenediaminetetraacetic acid (EDTA)) was incubated with each of the photoactivable TTX derivatives (1 μM) at 0 °C in the dark. After 30 min, a 200 μl aliquot of the incubation mixture was taken out to measure the complex formed by the rapid filtration method with a small column of Sephadex G-50 as described above. The residual sample was transferred equally to three plastic Petri dishes (for cell culture, 2.5 cm diameter) on ice and irradiated. The irradiation was carried out with a 40 W black lamp (Toshiba FL40S-BLB) at a distance of 5 cm for 10 min (TTX[^{3}H]enNAP and TTX[^{3}H]enTDB) or 30 min (TTX[^{3}H]en2FNP and TTX[^{3}H]en4FNP). In the separate experiments, it was confirmed that the photoactivable groups were photolyzed completely within the irradiation time. As controls, the samples in the presence of 100 μM unlabeled TTX were similarly incubated and irradiated. All the irradiated samples were heat-denatured at 100 °C for 2 min in the presence of 1% SDS and 1% 2-mercaptoethanol, followed by reductive carboxymethylation as described previously.[3] The samples were then fractionated on a column of Sepharose 4B-CL as described by Miller *et al.*[16] Eluted fractions were monitored by measuring radioactivity incorporated and by fluorescamine assay for proteins. Eluted sodium channel fractions of high molecular weight (*ca.* 250 kDa)[15,16] which were monitored by SDS-PAGE, were pooled and the photoincorporated amounts per sodium channel protein were determined. Photoincorporation yields (%) were calculated as the photoincorporated amounts of the Sepharose 4B-purified sodium channel/TTX[^{3}H]Lys sites of the sodium channel initially used.

4. Enzyme Digestions Photolabeled and pooled sodium channels after Sepharose 4B separation were further purified by gel-permeation chromatography on a column of TSK G4000 SW (7.5 × 60 cm) as described.[3] The purified sodium channel proteins were dialyzed and lyophilized and then digested with proteases. The TTX[^{3}H]enNAP-labeled channel protein (650 μg, 2.5 nmol) in 0.5 ml of 50 mM Tris–HCl (pH 9.0) was digested with lysyl endoprotease (Achromobacter protease I, Wako Chemical Co., Osaka) (S:E = 500, w/w) at 37 °C for 12 h. TTX[^{3}H]enTDB-labeled channel protein (2.7 mg, 10.4 nmol) in 1.1 ml of 50 mM Tris–HCl (pH 9.0) containing 10 mM $CaCl_2$ was digested with TPCK-trypsin (Worthington) (S:E = 100, w/w) at 37 °C for 12 h, followed by additional 12 h digestion after addition of an equal amount of the enzyme.

5. HPLC Separation of the Peptide Fragments Lysyl endoprotease-digest of the TTX[^{3}H]enNAP labeled sodium channel was loaded on a Novapak 5C18 column (3.9 × 150 mm, Waters) which was preequilibrated with 0.1% trifluoroacetic acid and developed with the same solvent over 10 min, followed by a linear gradient of acetonitrile–2-propanol (3:7, v/v) consisting of 0—10% over 10 min, 10—60% over 25 min, and 60—90% over 10 min, at a flow rate of 1 ml/min; Waters M510 pumps with a 680 Automated Gradient Controller were used. Fractions were monitored by measuring the absorbance at 215 nm with a Hitachi 638 UV monitor and the radioactivity of the fraction aliquots was counted. The fractions eluted at 33—34 min were pooled and the solvent was evaporated off *in vacuo*. The residue was dissolved in 0.1% trifluoroacetic acid (200 μl) and rechromatographed on the same column which had been pre-equilibrated with 10 mM ammonium acetate (pH 6.0) containing 5% acetonitrile, followed by a linear gradient of 5—30% acetonitrile over 30 min at a flow rate of 1 ml/min.

TPCK-trypsin digest of the TTX[^{3}H]enTDB labeled sodium channel was loaded on a μ-Bondasphere 5C4/300 Å column (3.9 × 150 mm; Waters) which had been pre-equilibrated with 0.1% trifluoroacetic acid and developed with a linear gradient of acetonitrile–2-propanol (3:7, v/v) consisting of 0—60% over 75 min, followed by 60—90% over 10 min at a flow rate of 0.5 ml/min. Monitoring of the eluates was similarly carried out by measuring of absorbance at 215 nm and the radioactivity.

Acknowledgement This work was supported by grants from the Ministry of Education, Science and Culture, the Life Science Research Project of the Institute of Physical and Chemical Research (Riken), the Mitsubishi Foundation, the Toray Science Foundation, the Naito Foundation, and the Fujisawa Foundation.

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