Biochimica et Biophysica Acta, 664 (1981) 219–225
Elsevier/North-Holland Biomedical Press

BBA 79259

SYNTHESIS AND PROPERTIES OF NEW PHOTOACTIVABLE DERIVATIVES OF TETRODOTOXIN

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(Received December 8th, 1980)

Key words: Tetrodotoxin derivative; Neurotoxin synthesis; Photoactivation

The Pfitzner-Moffatt oxidation procedure has been used to prepare two new photoactivable derivatives of tetrodotoxin that have been synthesized with high specific radioactivities (17.5 Ci/mmol and 30 Ci/mmol). They specifically bind to axonal membranes with affinities of 5.2–14.2 nM. They dissociate from their membrane complex with half-lives of 10.8 and 20 min. In the dark, these compounds give a reversible block of the sodium channels. After ultraviolet irradiation, they induce an irreversible blockade of the nerve channels.

Introduction

Many neurotoxins are known to specifically alter the functioning of the voltage-dependent sodium channel. They can be separated in four main groups based on their action on at least four different types of receptors [1]: (i) tetrodotoxin and saxitoxin which block Na^+ entry through the Na^+ channel without altering the gating mechanism of the channel [2,3]; (ii) batrachotoxin, veratridine, aconitine and grayanotoxin which alter both the activation and the inactivation of the Na^+ channel [4–7]; (iii) pyrethroids which inhibit sodium inactivation [8,9] and (iv) polypeptide toxins like scorpion and sea anemone toxins [10,11] which also inhibit the inactivation process. Tetrodotoxin is still the most widely used toxin in the analysis of the properties of the fast Na^+ channel. The receptor of this toxin has been biochemically identified in a variety of preparations of excitable membranes [3,12] and even partially purified [13–15].

Highly radioactive tetrodotoxin derivatives recently obtained by synthesis [16] have been found to associate to the tetrodotoxin-binding component and to exhibit biological properties similar to those of tetrodotoxin itself. Photoactivable derivatives of tetrodotoxin have also been synthesized; in the dark they give a reversible block of the sodium channel and this block becomes irreversible after ultraviolet irradiation [17,18]. The purpose of this paper is to describe the synthesis and the properties of new photoaffinity derivatives of tetrodotoxin which we find more useful than those which have been previously described.

Materials and Methods

Chemicals. Tetrodotoxin citrate-free was obtained from Sankyo Chemicals Co. 4-fluoro-3-nitroaniline, $NaCNBH_3$, DCCD and crystallized H_3PO_4 (99% purity) were purchased from Fluka AG. Dried Me_2SO, 2,4-dinitrophenylhydrazine, ethylenediamine, Silica Gel GF_{254} and cellulose plates were from Merck. All solvents were of the best analytical grade and they were dried in the presence of preactivated 3 Å molecular sieves. [^{3}H]Lysine (20 Ci/mmol), [^{3}H]ethylenediamine (30 Ci/mmol) were obtained from the Commissariat à l'Energie Atomique, France.

Synthesis. FNAB was prepared from 4-fluoro-3-nitroaniline according to Fleet et al. [19]. After recrystallization the product was checked for their purity by infra red and ultraviolet spectroscopy and stored at −15°C in the dark.

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Abbreviations: $NaCNBH_3$, sodium cyanoborohydride; DCCD, dicyclohexylcarbodiimide; Me_2SO, dimethyl sulfoxide; FNAB, 1-fluoro-2-nitro-4-azidobenzene; NAP, 2-nitro-4-azidophenyl.

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NAPethylenediamine was synthesized from FNAB and ethylenediamine as already described by Chicheportiche et al. [17]. The synthesis of the tritiated product was also carried out. For this purpose 12.5 mCi [^{3}H]ethylenediamine in 100 μl methanol were reacted with 400 μl 1.5 mM FNAB in Me_2SO. The reaction was started by addition of 0.1 μl triethylamine and allowed to proceed at 35°C for 16 h. After lyophilization the product was purified by chromatography on cellulose plate using acetonitrile/0.1 M ammonium formate, pH 4.5 (3 : 1) as a solvent. The component of $R_F = 0.77$ was eluted from the cellulose powder with 1 ml methanol and used for the coupling reactions. NAPethylenediamine (25% yield) had a specific radioactivity varying between 27.0 and 30.7 Ci/mmol as measured from its ultraviolet spectrum ($\epsilon_M^{450} = 5200\ M^{-1} \cdot cm^{-1}$, $\epsilon_M^{260} = 23\,500\ M^{-1} \cdot cm^{-1}$) and radioactivity.

NAPlysine was synthesized as for NAPethylenediamine in Me_2SO. The product obtained had the same ultraviolet and chromatographic characteristics as given by Smith and Knowles [20]. The tritiated compound was also synthesized. NAP[^{3}H]lysine was prepared from 25 mCi [^{3}H]lysine in 50 μl water and 3.5 mg FNAB in 3.8 ml Me_2SO. After addition of 1 μl triethylamine, the reaction was allowed to proceed for 8 h at 35°C. The medium was then lyophilized and the product purified by thin-layer chromatography on Silica Gel GF_{254} plate. A radioactive colored band comigrated with unlabeled NAPlysine ($R_F = 0.5$) in a chloroform/methanol/ammonia (45 : 45 : 10) system. The purified product was eluted from Silica Gel with 1 ml methanol/ammonia (9 : 1) lyophilized and redissolved in 1 ml methanol. NAP[^{3}H]lysine (45% yield) had a specific radioactivity of 17.5 Ci/mmol.

NAP[^{3}H]lysine and NAP[^{3}H]ethylenediamine were coupled to tetrodotoxin following the method described by Chicheportiche et al. [16] for the synthesis of highly radioactive tetrodotoxin derivatives. Briefly, 1 ml tetrodotoxin was oxidized by the Pfitzner-Moffatt method [21]. The aqueous phase containing oxidized tetrodotoxin was mixed with 50 nmol NAP[^{3}H]ethylenediamine or 175 nmol NAP[^{3}H]lysine in 1 ml methanol. After 2 h, the reaction mixture was lyophilized. The material was redissolved in 200 μl of a solution of 10 mM $NaCNBH_3$ in anhydrous methanol. The pH was adjusted to 6.0 with acetic acid and the reaction was allowed to proceed at 35°C for 3 h in the case of NAP[^{3}H]ethylenediamine or 60 h for NAP[^{3}H]lysine. The reaction was followed by bidimensional chromatography of 2 μl of the reaction mixture on a cellulose plate.

When chloroform/methanol (2 : 1) was used as a solvent in the first dimension, the unreacted NAP[^{3}H]ethylenediamine and NAP[^{3}H]lysine were resolved from the two NAPtetrodotoxin derivatives which remained at the starting point. The use of acetonitrile/0.1 M ammonium formate pH 4.5 (3 : 1) as the eluent in the second dimension, separated tetrodotoxin from NAP[^{3}H]ethylenediamine tetrodotoxin (colored spot at $R_F = 0.23$) or from NAP[^{3}H]lysine tetrodotoxin (colored spot at $R_F = 0.38$). The final one-step purification was carried out on cellulose plate using acetonitrile/0.1 M ammonium formate pH 4.5 (3 : 1). The yellow band so obtained was scraped off, extracted with methanol/water (1 : 1) and rechromatographed in the same system to give the desired product, namely NAP[^{3}H]ethylenediamine tetrodotoxin or NAP[^{3}H]lysine tetrodotoxin at $R_F = 0.31$ or 0.40, respectively.

Either purified product was extracted from cellulose with an 1 mM acetic acid solution and used for the biological experiments.

Electrophysiological measurements. The biological activities of tetrodotoxin and NAPtetrodotoxin derivatives were tested as already described [17] by their ability to inhibit the rising phase of the action potential of the giant axon isolated from the circumoesophageal nerve of the crab *Carcinus maenas.*

Photoirradiation. The irreversibility of the action of photoactivable tetrodotoxin derivatives was tested by electrophysiological techniques on the crustacean nerve after irradiation. The preparation positioned at 7 cm from the ultraviolet light (100 W) was flashed with a spectroflash switching unit (Applied Photophysics, London).

Axonal membranes. Purified axonal membranes were prepared from walking legs axon bundles of *Cancer pagurus* as previously described [22].

Binding assays. Binding measurements were performed at 4°C in 50 mM potassium phosphate buffer, pH 7.5. Increasing concentrations of NAP-tritiated ligands were incubated 20 min with purified crab axonal membranes and filtered on Millipore EGWP 0.22 μm filters as previously described [22]. As

usual, the unspecific binding was the binding of the tritiated NAP ligands which was not protected (or displaced) by an excess of unlabeled tetrodotoxin at a 5 μM concentration. Specific binding (the difference between total and unspecific binding) was found to be higher than 80% at concentrations corresponding to the value of the dissociation constant.

Dissociation kinetics. Dissociation kinetics were measured at 4°C. After 30 min incubation of the axonal membranes (0.1 mg protein/ml) with 10 nM NAP[^{3}H]ethylenediamine tetrodotoxin or NAP-[^{3}H]lysine tetrodotoxin, a 500-fold excess of unlabeled tetrodotoxin was added to the medium to displace the ^{3}H-labeled ligand from its binding site. This decrease of specific binding was followed by taking 100 μl aliquots at different time intervals. These aliquots were then filtered under the previously described conditions for binding assays.

Results

Synthesis and purifications of NAP[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin

The synthetic procedure used here was similar to that used for the synthesis of highly labelled tetrodotoxin derivatives [16]. Tetrodotoxin was oxidized by the Pfitzner-Moffatt reaction as previously described. The oxidation product was coupled to an amino-NAP derivative at a high specific radioactivity by reductive amination with $NaCNBH_3$ as the reductive agent. Fig. 1 shows the scheme of the reaction used with NAP-[^{3}H]ethylenediamine and NAP[^{3}H]lysine. The first step of the reaction involved the oxidation of tetrodotoxin to form a carbonyl function probably at the C_{11} position [16]. This function was easily detected as previously described [16]. The coupling reaction between NAP[^{3}H]ethylenediamine or NAP[^{3}H]lysine and oxidized tetrodotoxin required a step of Schiff-base formation followed by a reduction with $NaCNBH_3$ at pH 6.0–7.0 which is very selective for the reduction of the imino-function. Under these mild conditions, the biological activity of tetrodotoxin itself was not affected. Purifications of the NAPtetrodotoxin derivatives were achieved by bidimensional chromatography on cellulose plates. Using chloroform/methanol (2 : 1) as a solvent in the first dimension, we were able to separate the unreacted NAP-amino reactants from the products. The orange spot remaining at the origin was eluted in the second dimension (Fig. 2) with the system acetonitrile/0.1 M ammonium formate pH 4.5 (3 : 1). This second step gave colored spots for NAP[^{3}H]ethylenediamine tetrodotoxin and for NAP[^{3}H]lysine tetrodotoxin at $R_F = 0.23$ and 0.38, respectively, separated from unreacted tetrodotoxin and oxidized tetrodotoxin.

The excess of unreacted tetrodotoxin ($R_F = 0.15$) was totally eliminated from the NAPtetrodotoxin derivatives by rechromatography of the spots in the same second elution system to give single colored spots for NAP[^{3}H]ethylenediamine tetrodotoxin or NAP[^{3}H]lysine tetrodotoxin at $R_F = 0.31$ or 0.40, respectively.

The purified products had a total 20–25% yield as

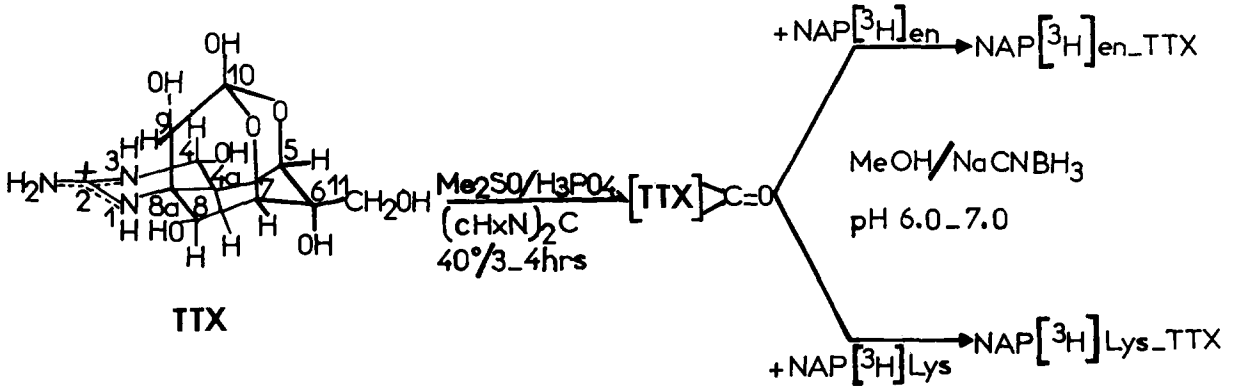

Fig. 1. Pathway of the synthesis of NAPtetrodotoxin derivatives with high specific radioactivity. TTX, tetrodotoxin; en, ethylenediamine; Lys, lysine; $(cHxN)_2C$, dicyclohexylcarbodiimide.

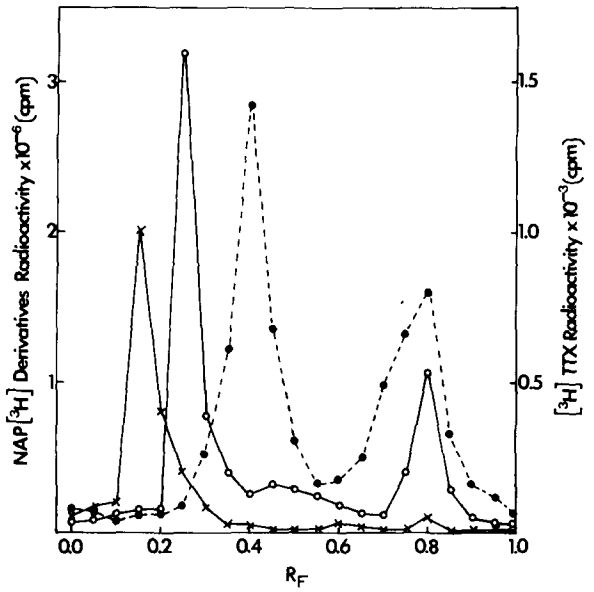

Fig. 2. Purifications of NAP[^{3}H]ethylenediamine tetrodotoxin (○———○) and NAP[^{3}H]lysine tetrodotoxin (●- - - - -●) by thin layer chromatography. Elution was performed from 2 μl of the reacting medium which was spotted on the cellulose plate using chloroform/methanol (2 : 1) in the first dimension and acetonitrile/0.1 M ammonium formate, pH 4.5 (3 : 1) in the second dimension. The second dimension is only represented. The colored spots comigrated with radioactivity with R_F = 0.23 and 0.40 for NAP[^{3}H]-ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin, respectively; the ones of R_F = 0.8 were inactive and corresponded to some unreacted NAP[^{3}H]ethylenediamine and NAP[^{3}H]lysine which had not migrated in the first dimension and/or to inactive tetrodotoxin adducts. In the same elution system, tritiated tetrodotoxin and oxydized tetrodotoxin (X———X) have a same R_F value of 0.15 (second dimension). Repurifications of NAP[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin gave active products free from unreacted tetrodotoxin.

compared to the starting tritiated NAPethylenediamine or NAPlysine and 1–2% yield as compared to the starting tetrodotoxin.

The identification of the spots as the NAPtetrodotoxin derivatives was based on the following: (a) characteristic orange color of the NAP group; (b) presence of radioactivity corresponding to the ethylenediamine or lysine moieties; (c) ability to specifically bind to the Na^+ channel of axonal membranes as it was demonstrated in the binding section, a property which is specific for the tetrodotoxin moiety; (d) the coupling stoichiometry between oxidized tetrodotoxin and NAP[^{3}H]ethylenediamine as determined by direct measurement of the tetrodotoxin moiety by the fluorescence method of Núñez et al. [23] and ourselves [16] and by the ultraviolet spectrum of the NAP moiety. A ratio of 0.92 : 1 was obtained between the fluorescence and ultraviolet data. On the basis of these results we then reasonably assumed that the 1 : 1 ratio was also applicable to the synthesis of NAP[^{3}H]lysine tetrodotoxin. The same ratio was also obtained for the coupling stoichiometry of [^{3}H]ethylenediamine tetrodotoxin [16].

Binding properties of NAP[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin

Fig. 3A shows the specific binding of the two NAPtetrodotoxin derivatives to purified axonal membranes at 4°C, pH 7.5 obtained by the rapid filtration method. The equilibrium dissociation constants were 14.2 and 5.2 nM with NAP[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin, respectively. The maximal binding capacities were 14.2 and 12.7 pmol/mg of protein, respectively, as previously determined with [^{3}H]saxitoxin or other tetrodotoxin derivatives using the same crab axonal membranes [16].

Dissociation kinetics are semilogarithmically presented in Fig. 3B. Rate constants of dissociation of $5.7 \cdot 10^{-4}$ s^{-1} and $10.6 \cdot 10^{-4}$ s^{-1} (4°C, pH 7.4) were found with complexes formed between crab axonal membranes, and respectively NAP[^{3}H]ethylenediamine tetrodotoxin or NAP[^{3}H]lysine tetrodotoxin. Half-lives of complex NAPtetrodotoxin-receptor were 20 and 10.8 min, respectively.

Biological activities and photoactivability of NAP-[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]-lysine tetrodotoxin

The biological activity of the NAPtetrodotoxin derivatives and their photoactivability were tested on crab nerves by their ability to decrease the rate of the rising phase of the action potential. Fig. 4A shows that an application of 20 nM NAP[^{3}H]lysine tetrodotoxin produces a blocking effect of the action potential which is reversible in the dark, and irreversible after ultraviolet irradiation even after a long period of washing. In control experiments made before any application of NAPtetrodotoxin derivative, this block

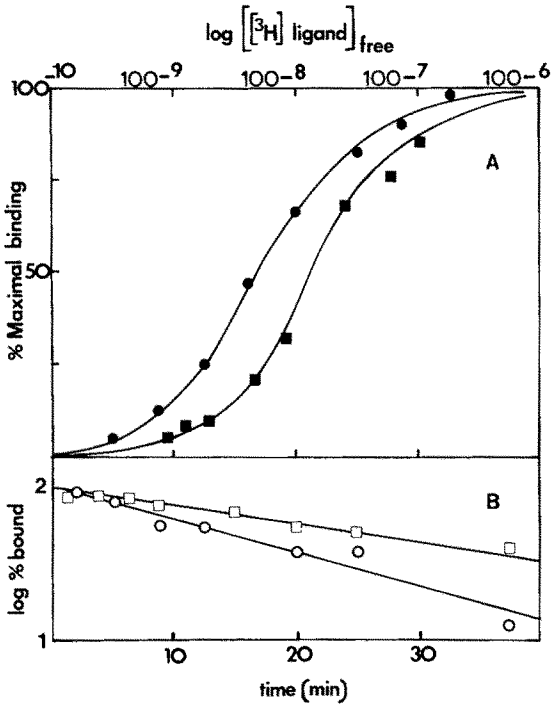

Fig. 3. Binding characteristics of NAP[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin on crab axonal membranes at 4°C. (A) Specific binding NAP[^{3}H]-ethylenediamine tetrodotoxin (▪) and NAP[^{3}H]lysine tetrodotoxin (•). Axonal membranes (200 μg) were incubated 30 min in 50 mM potassium phosphate buffer at pH 7.5 with increasing amounts of radioactive ligand (final volume = 500 μl). Binding measurements were carried out by rapid filtration of 2 · 200 μl of the incubation medium. Half-maximal binding (K_D values) was observed at 14.2 and 5.2 nM, and maximal binding capacities at 14.2 and 12.7 pmol/mg protein for NAP[^{3}H]ethylenediamine tetrodotoxin and NAP[^{3}H]-lysine tetrodotoxin, respectively. (B) Pseudo-first order representations of the dissociation kinetics of the NAP[^{3}H]-ethylenediamine tetrodotoxin-membrane complex (□) and the NAP[^{3}H]lysine tetrodotoxin-membrane complex (○). Radioactive ligand (10 nM) were incubated 30 min at 4°C with crab axonal membranes. Dissociation was started by the addition of 10 μM unlabelled tetrodotoxin. Replacement of NAP[^{3}H]ethylenediamine tetrodotoxin or NAP[^{3}H]lysine tetrodotoxin by unlabelled tetrodotoxin was followed by measuring the decrease of ^{3}H radioactivity bound to axonal membranes using rapid filtration technique.

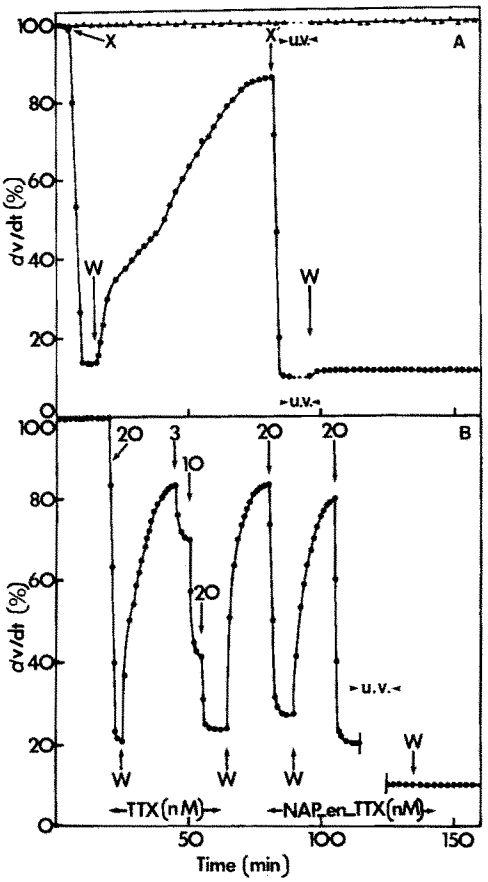

Fig. 4. Comparative effects of tetrodotoxin, NAP[^{3}H]-ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin on the rate of the rising phase of the action potential of crab giant axon. (A) Addition (X) of 20 nM NAP[^{3}H]lysine tetrodotoxin (•———•) induced inhibition of 85% of the *dv/dt* signal. This effect was reversible by washing (W) in the dark. The same amount of NAP[^{3}H]lysine tetrodotoxin (X') followed by 50 ultraviolet flashes produced an inhibition of 90% of the signal which was irreversible even after 50 min washing. A control experiment (▲———▲) was made in the same conditions without tetrodotoxin or tetrodotoxin derivative. The same procedure of washing and ultraviolet flashes was without effect on the *dv/dt* signal of the crab giant axon. Controls were also made to show that a region of the axon adjacent to the one which was photoinactivated but not exposed to the action of the NAP derivatives gave 100% of the *dv/dt* value. (B) Tetrodotoxin (20 nM) reversibly inhibited 80% of the *dv/dt* signal. After washing (W), the addition of 3, 10 and 20 nM tetrodotoxin produced an inhibition proportional to these concentrations. After a new washing (W), NAP[^{3}H]ethylenediamine tetrodotoxin (20 nM) produced the same reversible inhibitory effect in the dark. A new addition of this derivative (20 nM) followed by 50 ultraviolet flashes led to the block of 90% of the Na^+ channels that was persistant even after a prolonged washing (30 min).

Control made before and after these series of measurements are similar to those described with NAP[^{3}H]lysine tetrodotoxin. TTX, tetrodotoxin; en, ethylenediamine; U.V., ultraviolet.

of the Na^+ conductance was not seen with the same ultraviolet irradiation. Furthermore, a control of the excitability made in a region of the axon adjacent to the one which was photoinactivated, but not exposed to the action of the NAPtetrodotoxin derivative, gave 100% of the *dv/dt* value of the control. Crab axons were resistant to ultraviolet light under the experimental conditions used.

The same result was obtained with NAP[^{3}H]-ethylenediamine tetrodotoxin (Fig. 4B). In the dark, the inhibitory effect of 20 nM NAP[^{3}H]ethylenediamine tetrodotoxin on the action potential is similar to that obtained with the same concentration of tetrodotoxin. The blocking effect observed in the dark was reversible by washing. When the same concentration of photoactivable product was applied to the preparation, ultraviolet irradiation led to an irreversible block of more than 90% of the Na^+ channels even after prolonged washing.

Discussion

Two methods have been previously proposed to synthesize photoactivable derivatives of tetrodotoxin. The first method involved the direct coupling of a NAP-activated ester on alcohol functions on the tetrodotoxin molecule [18]. Unfortunately, we have been unable to reproduce this synthesis. The other method involves the coupling of NAP-amino derivatives to tetrodotoxin oxidized by periodate on the C_6-C_{11} position [17].

The new photoactivable compounds described here are more interesting than those previously described in that, (i) they have a higher affinity for the tetrodotoxin receptor, and (ii) they can be obtained in a tritiated form with a very high specific radioactivity (30 and 17.5 Ci/mmol for NAP[^{3}H]-ethylenediamine tetrodotoxin and NAP[^{3}H]lysine tetrodotoxin, respectively).

NAPethylenediamine tetrodotoxin and NAPlysine tetrodotoxin form complexes with the Na^+ channel which have dissociation constants of 14.2 and 5.2 nM. These dissociation constants are very close to those found for [^{3}H]ethylenediamine tetrodotoxin and [^{3}H]lysine tetrodotoxin which are 15 and 4.1 nM, respectively [16]. Under the same conditions tetrodotoxin itself forms a complex with a dissociation constant of 3.3 nM [16]. These data indicate that the bulky NAP group linked to tetrodotoxin does not modify the affinity of the toxin for its binding site. They also confirm that the C_6-C_{11} region of the tetrodotoxin molecule does not directly participate in the interactions between the toxin and its receptor site on axonal membranes. We can also remark that the half-lives (10 and 20 min) of complexes formed with the newly synthesized photoaffinity derivatives of tetrodotoxin are much larger than that of the nitrene radical ($t_{1/2} = 10^{-4}$ s [24]). This is a necessary condition for an extensive irreversible labelling of the receptor site after illumination.

These two new photoactivable tetrodotoxin derivatives are now investigated to identify the binding site of tetrodotoxin in the sodium channel.

Acknowledgements

The authors are very grateful to N. Alenda and Dr. M.C. Lenoir for expert assistance, to Professor A. Scanu for a careful reading of the manuscript and to C. Bonifacino for skilful technical help. This work was supported by the Commissariat à l'Energie Atomique, the Délégation Générale à la Recherche Scientifique et Technique (grant No. 79.7.0786), the Institut National de la Santé et de la Recherche Médicale (grant No. 80.30.10) and the Centre National de la Recherche Scientifique.

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