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SHORT COMMUNICATIONS

Toxin T_{4_6} from *Ptychodiscus brevis* (Formerly *Gymnodinium breve*) Enhances Activation of Voltage-Sensitive Sodium Channels by Veratridine

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SUMMARY

CATTERALL, W. A., AND M. RISK. Toxin T_{4_6} from *Ptychodiscus brevis* (formerly *Gymnodinium breve*) enhances activation of voltage-sensitive sodium channels by veratridine. *Mol. Pharmacol.* 19:345–348 (1981).

Ptychodiscus brevis toxin T_{4_6} markedly enhances persistent activation of voltage-sensitive sodium channels in neuroblastoma cells by veratridine. Half-maximal enhancement is observed at 50 ng of T_{4_6} per milliliter. At maximally effective concentrations, T_{4_6} has no effect on specific binding of ^{125}I-labeled scorpion toxin and slightly enhances specific binding of [^{3}H]saxitoxin. Thus, T_{4_6} enhances veratridine action but does not bind to any of the three neurotoxin receptor sites on the sodium channels that have been previously described. T_{4_6} may act at a new toxin-receptor site associated with voltage-sensitive sodium channels.

The dinoflagellate *Ptychodiscus brevis* (formerly *Gymnodinium breve* (1)) is responsible for numerous fish kills during its blooms ("red tides") along the coast of the Gulf of Mexico and for human toxicity due to ingestion of contaminated shellfish. Partially purified preparations of *P. brevis* toxins cause repetitive firing in the squid giant axon (2) and increase the frequency of action potentials in isolated crayfish nerve cord (3). These results suggest that the toxin(s) may alter the properties of voltage-sensitive ionic channels in nerve membranes. Recently, two nonprotein toxins from *P. brevis* have been purified to homogeneity and designated T_{4_6} and T_{4_7} on the basis of chromatographic properties (4). In this report, we describe our initial studies of the effect of T_{4_6}, the more potent of the two pure toxins, on voltage-sensitive sodium channels in cultured neuroblastoma cells.

Previous work reviewed in ref. 5 has shown that there are three separate receptor sites for neurotoxins associated with voltage-sensitive sodium channels. The first receptor site binds the inhibitors tetrodotoxin and saxitoxin, which block ion transport through the sodium channel. The second receptor site binds grayanotoxin and the alkaloids veratridine, batrachotoxin, and aconitine, which cause repetitive firing and persistent activation of sodium channels. The third receptor site binds the polypeptides scorpion toxin and sea anemone toxin, which slow sodium channel inactivation and enhance persistent activation of sodium channels by veratridine and other toxins acting at receptor site 2. Our approach in this study was to determine whether *P. brevis* toxin T_{4_6} modifies the interaction of neurotoxins with any of these three receptor sites defined in earlier work.

T_{4_6} is insoluble in aqueous medium. Preparation of stable aqueous solutions is therefore a significant technical problem. In the experiments presented here, stock solutions were prepared in 0.1% (v/v) Emulphor EL-620, an emulsifying agent (GAF), and diluted 10-fold into experimental solutions shortly before use. All solutions (including those for control cells) contained a final concentration of 0.01% (v/v) EL-620. The effect of this concentration of emulsifying agent on the experimental measurements is discussed below.

The passive sodium permeability of cultured neuroblastoma cells was determined by measuring $^{22}Na^+$ influx as described previously (6, 7) and in the legend to Fig. 1. T_{4_6} was incubated with neuroblastoma cells for 20 min at 36° in sodium-free standard binding medium at a resting membrane potential of −41 mV (7). The effect of the toxin on sodium permeability was then determined in a second incubation of 30 sec at 36° in assay medium containing the same T_{4_6} concentration, 10 mM ^{22}NaCl, and 5 mM ouabain to inhibit the sodium pump. These procedures give an accurate measure of passive sodium

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permeability at constant membrane potential (7). Under these conditions, T_{4_6} alone has no effect on the sodium permeability of N18 cells (Fig. 1, ●). Thus, T_{4_6} does not cause persistent activation of voltage-sensitive sodium channels in N18 cells. The closely related toxin T_4, caused a biphasic increase in $^{22}Na^+$ uptake in a human neuroblastoma clone (8).

Veratridine and related toxins cause persistent activation of sodium channels in neuroblastoma cells (7). Veratridine is a partial agonist in this respect, activating 8% of sodium channels at saturation (7). The addition of a saturating concentration of veratridine (200 μM) to the assay medium causes a small increase in $^{22}Na^+$ uptake by neuroblastoma cells (Fig. 1, ○, symbols on the ordinate) which is blocked by 1 μM tetrodotoxin (Fig. 1, △, symbols on the ordinate). If the neuroblastoma cells are first incubated with T_{4_6} and then $^{22}Na^+$ uptake is measured in the presence of 200 μM veratridine and T_{4_6}, a marked enhancement of veratridine action is observed (Fig. 1, ○). The initial rate of $^{22}Na^+$ influx is increased 8-fold with half-maximal effect at 50 ng of T_{4_6} per milliliter. The increase in $^{22}Na^+$ influx caused by veratridine and T_{4_6} is completely blocked by 1 μM tetrodotoxin (Fig. 1, △). These results show that T_{4_6} markedly enhances persistent activation of voltage-sensitive sodium channels by veratridine but does not cause persistent activation of a detectable number of sodium channels by itself. In other experiments (not shown), we have found that T_{4_6} also enhances activation of sodium channels by the full agonist batrachotoxin and by aconitine, a partial agonist at neurotoxin receptor site 2 of the sodium channel.

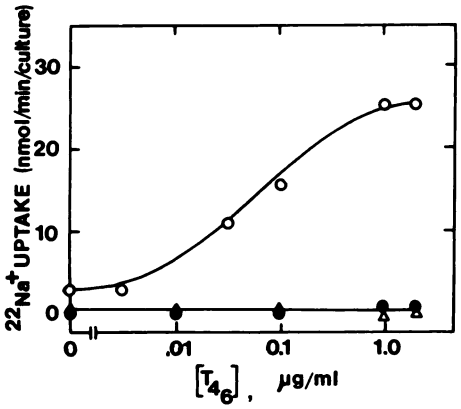

FIG. 1. *Concentration dependence of T_{4_6} effect on $^{22}Na^+$ influx*

Clone N18 neuroblastoma cells were grown in multiwell dishes as previously described (6). The cells in monolayer culture were incubated at 36° for 20 min with the indicated concentrations of T_{4_6} in 250 μl of standard binding medium consisting of 130 mM choline chloride; 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (adjusted to pH 7.4 with Tris base); 5.5 mM glucose; 0.8 mM $MgSO_4$; 5.4 mM KCl; bovine serum albumin, 1 mg/ml; and 0.01% (v/v) EL-620. This medium was removed by aspiration and the initial rate of $^{22}Na^+$ influx was measured during a 30-sec incubation at 36° in assay medium containing the same T_{4_6} concentration, 10 mM $^{22}NaCl$ (1 μCi/ml), 120 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, 0.01% (v/v) EL-620, and 5 mM ouabain with either no addition (●), 200 μM veratridine (○), or 200 μM veratridine plus 1 μM tetrodotoxin (△). Extracellular $^{22}Na^+$ was removed with three washes in 10 sec with wash medium (6) at 0°. The cells were dissolved in 0.4 N NaOH, and $^{22}Na^+$ influx was determined as described previously (6). $^{22}Na^+$ influx in the absence of added toxins (3 nmoles/min/culture) is subtracted from all data presented.

In the experiment presented in Fig. 1, all solutions contained a final concentration of 0.01% EL-620. This concentration of EL-620 causes a 20%–40% reduction in veratridine-stimulated $^{22}Na^+$ influx but has no effect on ^{125}I-labeled scorpion toxin or [^3H]saxitoxin binding as measured in the experiments of Fig. 3 and 4 below. In other experiments (not shown), we dissolved T_{4_6} in acetonitrile and diluted 100-fold into experimental medium immediately before use. In these experiments, T_{4_6} increased $^{22}Na^+$ influx to 25 nmoles/min/culture, as when dissolved in 0.01% EL620. However, a concentration of 400 ng of T_{4_6} per milliliter was required to give a half-maximal effect. It is likely that some T_{4_6} precipitates when experimental solutions are made by dilution from an organic solvent in this way. Therefore, in this report, we have included only results obtained with T_{4_6} solutions prepared with EL-620. The experiments with T_{4_6} dissolved in acetonitrile confirm that none of the effects observed are due to the use of EL-620 to solubilize T_{4_6}.

The time course of T_{4_6} action is illustrated in Fig. 2. In the presence of 200 μM veratridine, the onset of action of 200 ng of T_{4_6} per milliliter is rapid, reaching equilibrium within 5 min (Fig. 2, *left*, ○). The activation of sodium channels by veratridine alone was constant during this incubation time (Fig. 2, *left*, ●). In similar experiments (not shown) in which veratridine was not present during the first incubation with T_{4_6}, a rapid onset of T_{4_6} action was also observed. Thus, T_{4_6} interacts with the sodium channel rapidly in the presence or absence of veratridine and enhances veratridine activation.

The rate of reversal of action of 200 ng of T_{4_6} per milliliter is illustrated in the *right* panel of Fig. 2. The

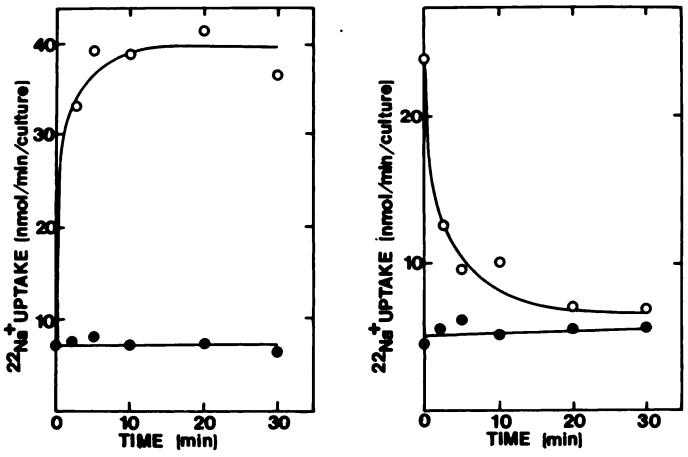

FIG. 2. *Time course of onset and reversal of T_{4_6} action*

Left. N18 cells were incubated at 36° with (○) or without (●) 200 ng of T_{4_6} per milliliter in standard binding medium containing 200 μM veratridine for the times indicated. $^{22}Na^+$ influx was then measured for 30 sec in assay medium containing 200 μM veratridine with (○) or without (●) 200 ng of T_{4_6} per milliliter as described in the legend to Fig. 1.

Right. N18 cells were incubated for 20 min at 36° with (○) or without (●) 200 ng of T_{4_6} per milliliter in standard binding medium containing 200 μM veratridine. The cells were then rinsed and incubated for the indicated times at 36° in standard binding medium containing 200 μM veratridine and no T_{4_6}. Finally, $^{22}Na^+$ influx was measured in the presence of 200 μM veratridine as described in the legend to Fig. 1. $^{22}Na^+$ influx in the absence of toxins (3.8 nmoles/min/culture) has been subtracted from the data.

effect of T_{4_6} is rapidly reversed with a half-time of 1–2 min (Fig. 2, *right*, ○). The rate of $^{22}Na^+$ influx approaches the value for veratridine alone (●) after 20 min. The interaction of T_{4_6} with the sodium channel therefore has a rapid rate of both onset and reversal.

The characteristics of T_{4_6} action described above are the same as those observed previously for the polypeptides scorpion toxin and sea anemone toxin (6, 9). Both of these toxins enhance alkaloid toxin activation of sodium channels but do not cause persistent activation themselves. The two polypeptide toxins act at a common receptor site associated with sodium channels (9). In order to test whether T_{4_6} also acts at this receptor site, we studied the effect of T_{4_6} on specific binding of ^{125}I-labeled scorpion toxin measured as described previously (10). These experiments were carried out on the same set of cell cultures as was the experiment illustrated in Fig. 1. The experimental procedure is described in the legend to Fig. 3. At concentrations of T_{4_6} up to 2 μg/ml, no effect on ^{125}I-labeled scorpion toxin binding is observed (Fig. 3), although the enhancement of veratridine activation is completed within this concentration range (Fig. 1). We conclude that T_{4_6} does not bind to the polypeptide toxin receptor site (receptor site 3) in causing enhancement of veratridine activation.

We also tested the effect of T_{4_6} on specific binding of [^3H]saxitoxin to its receptor site on the sodium channel measured as described previously (11). The method used is described in the legend to Fig. 4. The data of Fig. 4 show that, at concentrations that are effective in enhancing veratridine activation (Fig. 4, ●), T_{4_6} does not block [^3H]saxitoxin binding (Fig. 4, ○). In fact, a small enhancement of [^3H]saxitoxin binding was observed. Thus, T_{4_6} does not bind at any of the three neurotoxin receptor sites described in previous studies.

Taken together, our results show that T_{4_6} markedly enhances veratridine activation of sodium channels but does not bind to any of the previously described neurotoxin receptor sites. The T_{4_6} effect is half-maximal at 50

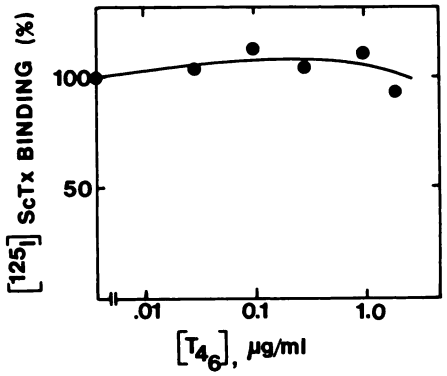

FIG. 3. *Effect of T_{4_6} on binding of ^{125}I-labeled scorpion toxin (ScTx)*
Scorpion toxin was purified (6) and radioactively labeled by lactoperoxidase-catalyzed iodination (10) as described previously. N18 cells were incubated for 60 min at 36° with 0.2 nM ^{125}I-labeled monoiodoscorpion toxin in standard binding medium containing the indicated concentrations of T_{4_6}. The cells were washed with wash medium (6) at 36°, and bound ^{125}I-labeled scorpion toxin was measured as described previously (10). Nonspecific binding measured in the presence of 200 nM unlabeled scorpion toxin (9% of total binding) has been subtracted from the data. Scorpion toxin binding measured in this way has been shown to represent specific binding to sodium channels (10).

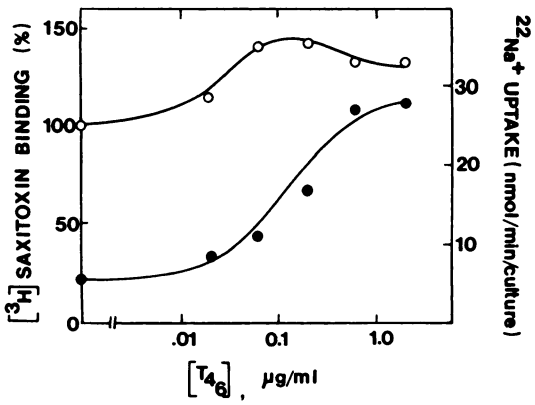

FIG. 4. *Effect of T_{4_6} on [^3H]saxitoxin binding*
Saxitoxin obtained from the National Institutes of Health was labeled with ^3H by the specific ^3H exchange procedure of Ritchie *et al.* (12) and was purified and characterized as described previously (13). The [^3H]saxitoxin used was 87% pure and had a specific radioactivity of 18.6 Ci/mmole. N18 cells were incubated for 20 min at 36° with 3 nM [^3H]saxitoxin in standard binding medium containing the indicated concentrations of T_{4_6}. The cells were washed with wash medium (6) at 0°, and bound [^3H]saxitoxin (○) was measured as described previously (11). Nonspecific binding in the presence of 1 μM tetrodotoxin (28% of total binding) has been subtracted from the results. Saxitoxin binding measured in this way has been shown to represent specific binding to sodium channels (11). The effect of T_{4_6} on veratridine activation of $^{22}Na^+$ influx in companion cell cultures was determined as in legend to Fig. 1 and is presented for comparison (●).

ng/ml (Fig. 1). From plasma desorption mass spectrometry, the molecular weight of T_{4_6} has been shown to be 866 (14). Half-maximal effects on neuroblastoma cells are therefore observed at 58 nM. Since the toxin is active at a low concentration, it is likely that it acts at a specific receptor site on the sodium channel rather than by a more indirect mechanism such as alteration of general membrane properties. Our working hypothesis, therefore, is that T_{4_6} is the first ligand described for a fourth neurotoxin receptor site associated with voltage-sensitive sodium channels. Further work is required to confirm this hypothesis and to define the allosteric interactions between this new toxin receptor site and those previously described in terms of our allosteric model of neurotoxin action on voltage-sensitive sodium channels (7). T_{4_6} may provide a new tool that will be useful in analysis of the molecular properties of voltage-sensitive sodium channels.

The concentration of T_{4_6} required for a half-maximal effect on veratridine activation in neuroblastoma cells (50 ng/ml) is 10-fold higher than the LD_{50} in fish toxicity tests [5 ng/ml (4)]. It is not unusual for LD_{50} and K_D at a receptor site to differ by 10-fold or more. Such a difference might be due to species differences or to the requirement for modification of only a fraction of the sodium channels for a lethal effect. The action of T_{4_6} on sodium channels is therefore likely to be a significant component of the toxicity of T_{4_6}. It will be of great interest to study the effects of T_{4_6} on sodium channel function directly by voltage clamp experiments in the absence of veratridine. Presumably, enhancement of veratridine activation as measured in these experiments is an indirect reflection of toxic modifications of kinetic and/or voltage-dependent properties of sodium channels.

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