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The actions of a red tide toxin from *Ptychodiscus brevis* on single sodium channels in mammalian neuroblastoma cells

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The actions of brevetoxin (PbTX-3) were studied on single, voltage-dependent sodium channels and whole-cell currents from the neuroblastoma × glioma cell line NG108-15. Purified PbTX-3 shifted the activation of sodium channels to membrane potentials negative to normal. PbTX-3 did not alter the single-channel mean open lifetime, suggesting that the toxin does not change the rate of sodium channel inactivation from the open state. There was also no change in single-channel conductance. These results indicate that brevetoxin increases sodium current at rest by shifting the voltage dependence of channel activation and that the resulting depolarization is limited by channel inactivation.

Brevetoxin; Na^+ channel; Patch-clamp; (Neuroblastoma cell)

1. INTRODUCTION

The brevetoxins are a group of polyether neurotoxins isolated from the marine dinoflagellate *Ptychodiscus brevis*. In vitro application of PbTX-2 ('T34', 'GB-2', or 'BTX-B') or PbTX-3 ('T17' or 'GB-3') [1] to frog or rat neuromuscular preparations first increases the rate of spontaneous transmitter release [2,3] and then causes failure of neuromuscular transmission [4], effects that parallel those seen in isolated crayfish and squid nerves [5]. Application of PbTX-2 or PbTX-3 to voltage-clamped squid axons shifts activation of sodium currents to more negative membrane potentials and appears to increase the 'non-inactivating' component of the sodium current, with no effect on potassium currents [3,5]. These experiments suggested that brevetoxin modifies both activation and inactivation of sodium channels. In squid axons, PbTX-3 has also been reported to produce a marked slowing of the sodium channel kinetics [6].

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Conclusions regarding brevetoxin-induced modifications of inactivation rest principally on the observed increase in the 'non-activating' component of the sodium current in squid axon. The conclusions may not be completely valid for two reasons. First, the non-inactivating current (the part of the sodium current that does not shut off within a few milliseconds after strong depolarization) is a peculiar feature of squid axons that is not normally found in most nerves and muscles [7]. Second, block of inactivation is inconsistent with the limited depolarizations produced by brevetoxin when compared to the complete depolarizations observed in batrachotoxin (which clearly blocks inactivation) [8]. A better method for testing the actions of brevetoxin on inactivation is to measure the open lifetime of single sodium channels. This is based on the notion that sodium channels opened by strong depolarization (e.g. ≥ -20 mV) will normally be closed by inactivation [9,10]. Thus, the inactivation rate can be deduced directly from the reciprocal of the channel open time. Consequently, we have examined the actions of brevetoxin at single sodium channels in a model system that has well characterized sodium currents.

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2. MATERIALS AND METHODS

Neuroblastoma × glioma hybrid NG108-15 cells were grown under standard conditions in DMEM and 5% fetal bovine serum, supplemented with 100 μM hypoxanthine, 1 μM aminopterin and 16 μM thymidine. To increase the density of sodium channels, cells were treated with 1 mM dibutyryl cyclic AMP for 4–10 days prior to electrophysiological studies.

Patch-clamp micropipettes were pulled from Kimax-51 glass and had resistances of 4–8 MΩ. The electrodes contained an 'internal' solution consisting of (mM) CsCl (140), NaCl (5), $MgCl_2$ (1), EGTA (5) and Hepes (5) buffered to pH 7.4. Whole cells and the outer surface of excised outside-out patches were bathed in an 'external' patch solution consisting of (mM) NaCl (140), KCl (5), $CaCl_2$ (1.8), $MgCl_2$ (1), tetraethylammonium (TEA^+) chloride (10) and Hepes (10) at pH 7.35. Purified PbTX-3 was obtained from the US Army Medical Research Institute for Infectious Diseases (Ft. Detrick, MD) and dissolved in $CHCl_3$. Working concentrations of the toxin were prepared by adding an appropriate amount of the toxin directly to the saline solutions yielding a total $CHCl_3$ concentration of less than 0.01% (v/v). Identical amounts of vehicle were added to the control solutions. Vehicle alone had no detectable effect on any measured parameter of channel function.

Recordings were performed at room temperature (21–25°C) using a List EPC-7 patch-clamp amplifier. Currents were low-pass filtered with an 8-pole Bessel filter at 5 kHz, digitized at 40-μs intervals and stored for off-line analysis using the version 5.0 of the pClamp software package [11]. Capacitative transients and leakage currents were removed by analog and digital subtraction. The nomenclature for the patch configurations follows that of Hamill et al. [12] and all membrane voltages are referenced to bath potential.

3. RESULTS

Resting membrane potentials in NG108-15 cells were measured under current clamp conditions. Bath application of 1.2 μM PbTX-3 depolarized the cells from -51.2 ± 2.3 mV (mean ± SE, $n = 12$) to -42.8 ± 0.4 mV ($n = 11$, $p < 0.005$). This depolarization was partially reversed by applica-

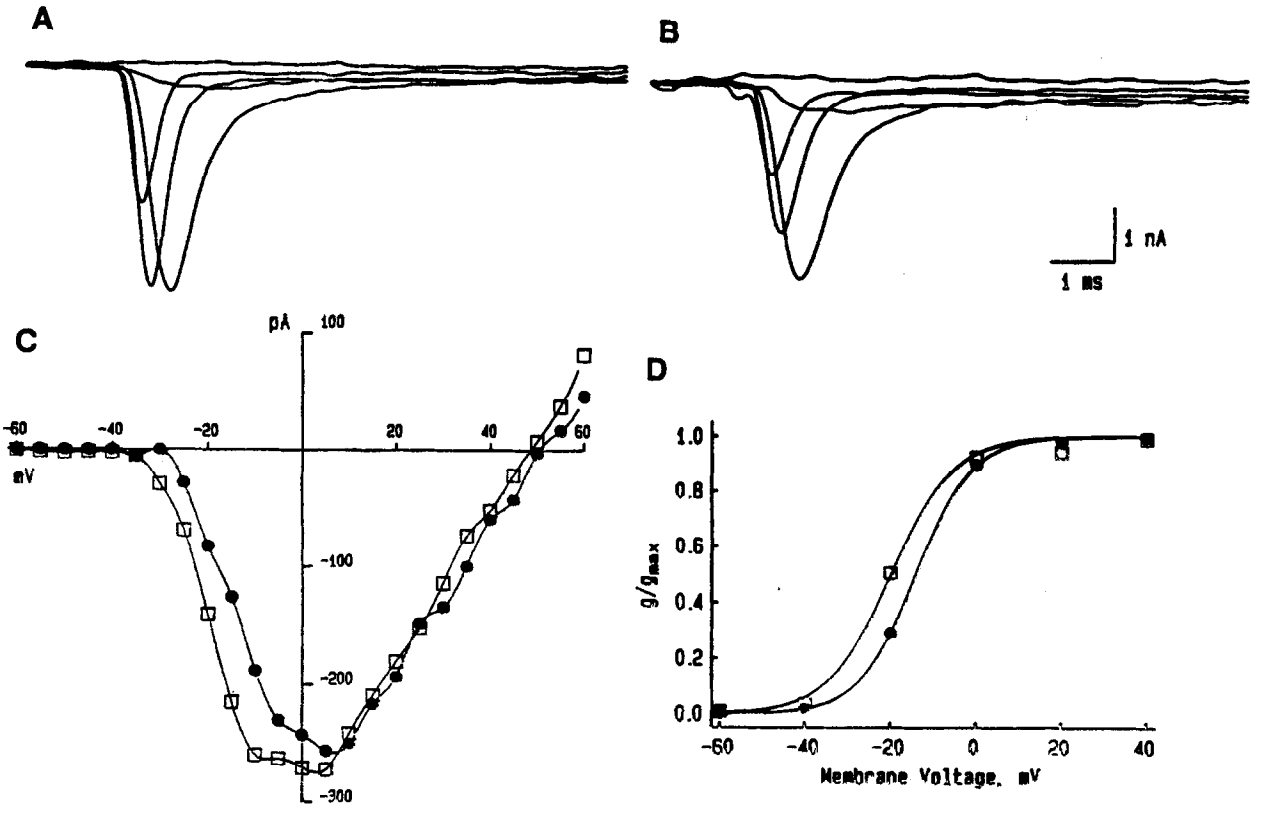

Fig.1. Whole-cell patch-clamp recordings of sodium currents before (A) and after (B) treatment with 2.5 μM PbTX-3. To obtain these records, the cell was held at −120 mV and stepped to −50, −30, −10, 10 and 30 mV. (C) Peak sodium currents are plotted as a function of membrane voltage in the absence (●) and presence (□) of 1.2 μM PbTX-3. (D) Plots of the negative shift in normalized peak conductance vs membrane voltage induced by 1.2 μM PbTX-3 (□). The fitted curve is a Boltzmann function with a voltage dependence of e-fold per 5.6 mV.

tion of 0.4 μM tetrodotoxin, returning the mean resting potential to -45.6 ± 1.4 mV ($n = 5$, $p < 0.01$ vs PbTX-3 treatment). Higher concentrations of PbTX-3 produced no additional effect, indicating that brevetoxin levels ≥ 1 μM are saturating [5]. Similar applications of 1 μM batrachotoxin (BTX) depolarized cells from -53.1 ± 2.0 mV (mean $\pm$ SE, $n = 8$) to 8.6 ± 1.0 mV ($n = 5$, $p < 0.0001$).

PbTX-3 shifted the activation of sodium channels to more negative potentials, as shown in the whole-cell currents of fig.1. Potassium currents were effectively blocked by the combination of internal Cs^+ and external TEA^+. In the presence of 1–7 μM brevetoxin, sodium activation shifted by -6.0 ± 1.7 mV (mean $\pm$ SE, $n = 7$), $p < 0.005$ in a paired t-test. No difference was observed between bath application and intracellular perfusion of the PbTX-3 via the patch electrode, with the maximal shift being produced within 5 min by either method. There was also no significant toxin-induced change in the reversal potential for the sodium currents (fig.1C). Further, the kinetics of the sodium currents remained essentially normal and no slow activation or inactivation of sodium current was observed in cells pulsed from a holding potential of -150 mV in 200–600 ms steps to -20 or -10 mV.

Typical recordings of single sodium channels in membrane patches excised from NG108-15 cells are shown in fig.2. Single sodium channel openings in the absence or presence of 20 μM PbTX-3 were similar. The major difference appeared to be a slightly higher incidence of channel opening in patches exposed to PbTX-3 at potentials near -50 to -60 mV. PbTX-3 did not increase the opening

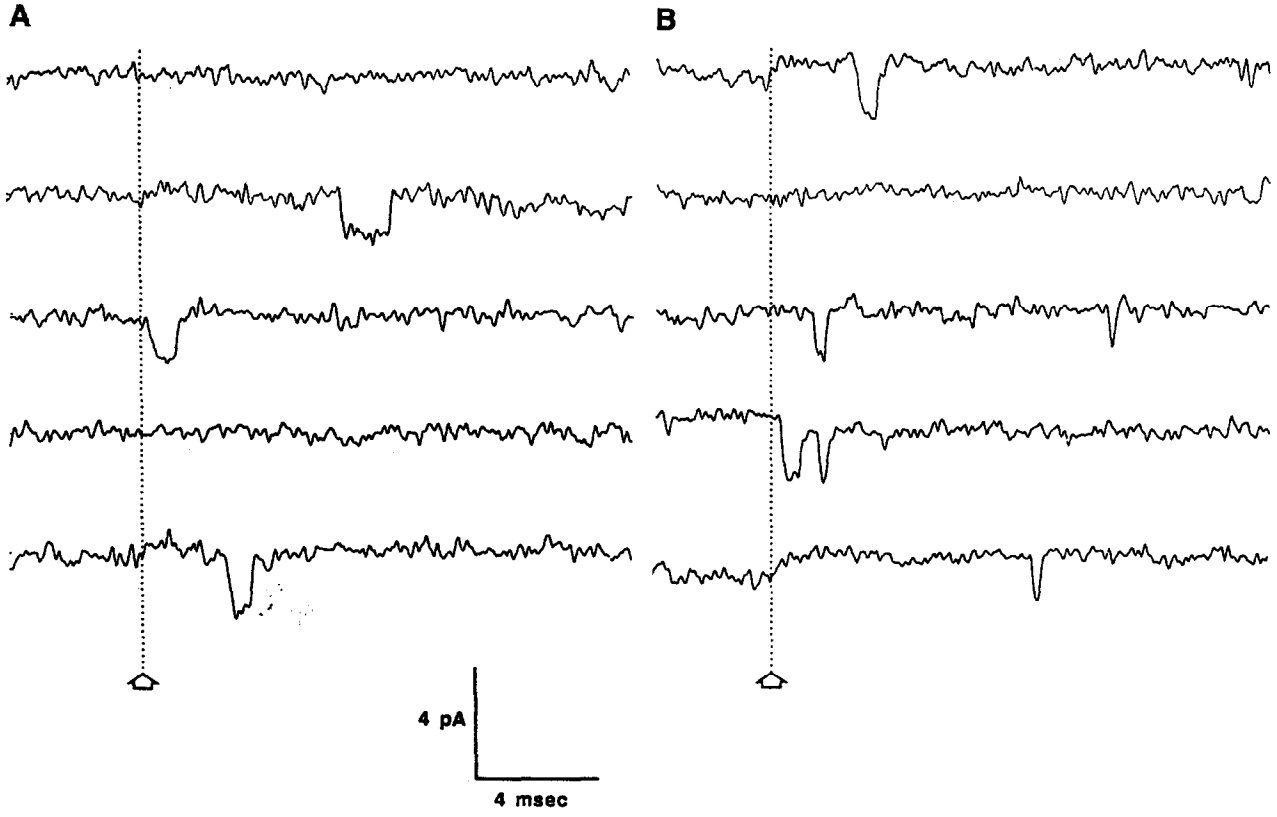

Fig.2. Single sodium channel currents from outside-out patches recorded under control conditions (A) and in the presence of 20 μM PbTX-3 (B). For each trace, the membrane was held at -90 mV and stepped to -40 mV at the time indicated by the dotted line. Both membrane patches contained at least three sodium channels.

probability of sodium channels at potentials between 0 and −30 mV, measured as the (number of channel openings)/(number of voltage steps) × (maximum number of channels seen in the patch). This fraction of channels opened per voltage step was 0.221 ± 0.032 (mean ± SE, 9 patches) in control and 0.222 ± 0.039 (5 patches) in PbTX-3.

Unlike sodium channel activation, channel inactivation was not changed by PbTX-3. Fig.3 shows histograms of open channel lifetimes under control conditions and in the presence of 1.2 μM PbTX-3 at −20 mV. In each case the histogram could be fitted with a single-exponential distribution, indicating a single, kinetically identifiable open state. At this potential, sodium channel openings are thought to be terminated by entry into one or more inactivated states and the lifetime of the open channel is inversely proportional to the rate at which channels 'inactivate' [9,10,13]. As seen in fig.3, the open state lifetime for channels at −20 mV did not change significantly during exposure to the same concentrations of PbTX-3 that altered the activation voltages (fig.1). Therefore, brevetoxin did not change the rate of sodium channel inactivation from the open state.

As has been described in other neuroblastoma cells, the open sodium channel lifetime did not vary with membrane voltage between −40 and 0 mV in NG108-15 cells. This phenomenon has been attributed to weak voltage dependence in inactivation rates [10] and supports the conclusion that our data at −20 mV are in the voltage range where inactivation is the major pathway for channel closure. Since there was little change in the maximum probability of channel opening, it seems likely that PbTX-3 also does not block inactivation from closed states. Otherwise, the probability of opening would have increased.

PbTX-3 did not change sodium channel conductance. Mean channel currents at −40 mV were −1.57 ± 0.022 pA (± SE) in control membrane patches and −1.61 ± 0.032 pA (± SE) in those modified by PbTX-3. There were no indications in the individual single channel records of multiple conductance states before or after treatment with the toxin, and the channel current distrubitions also indicated a single conducting state. Since the whole-cell records of fig.1C show no change in reversal potential, we conclude that there was no change in the single-channel conductance.

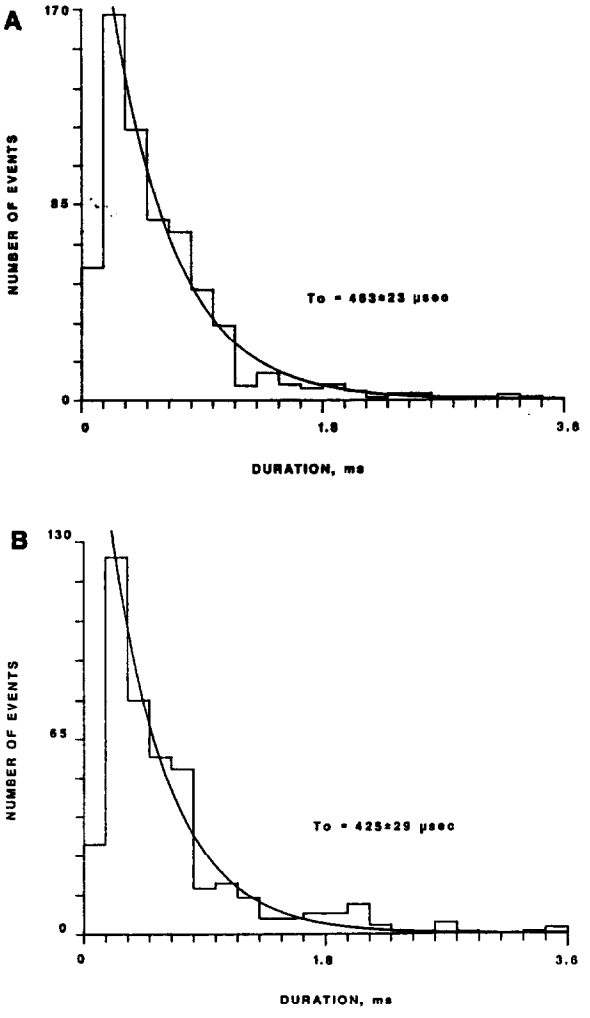

Fig.3. Histograms of open channel lifetimes recorded from membrane patches under control conditions (A) and after treatment with 1.2 μM PbTX-3 (B). The histograms were fitted with single-exponential distributions having the indicated time constants (T_o). Each histogram is a composite of 9–12 excised outside-out patches at −20 mV. The first sampling is underrepresented due to signal filtering.

4. DISCUSSION

Our results confirm previous findings that brevetoxin shifts the voltage dependence of sodium channel activation to more negative values [3,5]. This shift, although small, would be expected to have a profound effect on the resting membrane potential. Under normal resting conditions, the dynamic equilibrium between open, closed and in-

activated sodium channels could potentially lead to spontaneous depolarizations if it were not limited by channel inactivation. In the presence of brevetoxin, sodium channel activation is shifted to a range where the balance of currents in the membrane favors the recruitment of more open sodium channels and further depolarization. However, a balance is still produced by sodium channel inactivation that must result in less depolarization than is produced by an agent such as batrachotoxin, which demonstrably inhibits channel inactivation at the single-channel level [14]. This argument is supported by the much larger depolarizations seen in batrachotoxin-treated squid axons [8] relative to those produced by brevetoxin in squid and crayfish axons [5,15] as well as by our results in NG108-15 cells.

In summary, we feel that the actions of brevetoxin on electrically excitable sodium channels can best be described in terms of a negative shift in the voltage dependence of channel activation, with no effect on channel inactivation. This makes the physiological actions of brevetoxin distinct from other toxins, such as batrachotoxin, that both shift activation and remove (or slow) inactivation. This interpretation is further strengthened by the observation that brevetoxin and batrachotoxin bind to different sites on the sodium channel [16–18] in rat brain synaptosomal preparations. Thus, the biochemical classification of a unique brevetoxin-binding site on the sodium channel is confirmed by the distinct physiological properties of brevetoxin.

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