Modification of single Na⁺ channels by batrachotoxin
(patch clamp/neuroblastoma/channel conductance/channel lifetime)

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ABSTRACT  The modifications in the properties of voltage-gated Na⁺ channels caused by batrachotoxin were studied by using the patch clamp method for measuring single channel currents from excised membranes of N1E-115 neuroblastoma cells. The toxin-modified open state of the Na⁺ channel has a decreased conductance in comparison to that of normal Na⁺ channels. The lifetime of the modified open state is drastically prolonged, and channels now continue to open during a maintained depolarization so that the probability of a channel being open becomes constant. Modified and normal open states of Na⁺ channels coexist in batrachotoxin-exposed membrane patches. Unlike the normal condition, Na⁺ channels exposed to batrachotoxin open spontaneously at large negative potentials. These spontaneous openings apparently cause the toxin-induced increase in Na⁺ permeability which, in turn, causes membrane depolarization.

Batrachotoxin (BTX) is an extremely toxic steroidal alkaloid isolated from the skin of the Colombian arrow poison frog (1). It modifies the voltage-gated Na⁺ channel in excitable membranes and has been widely utilized to probe the properties of this class of channel (2, 3). Khodorov et al. have made extensive electrophysiological observations of the action of BTX on Na⁺ channels in the node of Ranvier (4–6). In these studies, BTX shifted Na⁺ channel activation to more negative potentials, and the kinetics of activation of the toxin-modified channel were slowed and inactivation was eliminated. Similar observations recently have been made for the actions of BTX on Na⁺ currents in neuroblastoma cells (7) and in squid axons (unpublished observations). BTX-modified channels were also less selectively permeable to Na⁺ over K⁺, Rb⁺, or Cs⁺ than normal channels. Further, the maximal Na⁺ permeability, if all voltage-gated channels were open, was substantially decreased after exposure to BTX. BTX also causes nerve and muscle membranes to depolarize due to an increase in resting Na⁺ permeability (8, 9).

These observations on the actions of BTX were primarily based on measurements of membrane currents recorded from the whole preparation by using conventional voltage clamp techniques. However, such currents represent the average behavior of a large population of channels of unknown number. Many questions remain regarding the action of BTX. Either reductions in the rate of channel closing or changes in the probability of channel opening could underlie the apparent lack of inactivation. The observed decrease in the maximal permeability for voltage-gated Na⁺ channels could be caused either by a block of Na⁺ channels or a reduction in the Na⁺ permeability for each modified channel. Modification of Na⁺ channels might be either in a graded or in an all-or-none manner. Further, changes in gating of the channel could be coupled to the changes in the permeation properties of the channel, or the two effects could be independent. Recently it has become possible to measure the current through individual voltage-gated Na⁺ channels (10–12). Changes in the fundamental properties of the Na⁺ channel that are modified by the toxin can be measured directly by using this approach.

N1E-115 neuroblastoma cells were utilized in this study because this preparation has a number of advantages for single channel studies. (i) There is free access to the plasma membrane, permitting a good seal between the membrane and the patch pipette. (ii) The cells can be voltage clamped and perfused internally to measure the average behavior of the population of channels. (iii) The Na⁺ channel density is low, permitting the measurement of individual single channel currents. The maximal Na⁺ conductance is about 17 mmho/cm² (10°C). Single Na⁺ channel conductance is not less than 10 pS at this temperature so that channel density is <17/μm². (iv) Na⁺ channel kinetics measured by whole cell voltage clamp techniques are markedly slower than those of many vertebrate or invertebrate preparations at the same temperature (13), thus decreasing the required temporal resolution in the recording system.

METHODS AND MATERIALS

N1E-115 neuroblastoma cells were maintained in tissue culture and grown in Dulbecco's modified Eagle's medium, supplemented with 10% newborn calf serum at 37°C in humidified air containing 10% CO₂. Three days to 2 wk before their use in this study, cells were first grown on coverslips in media to which 2% dimethylsulfoxide had been added to enhance the expression of neuronal characteristics (14).

The gigahorn-seal single-channel recording technique (15) was used to record currents from isolated membrane patches. Except where otherwise noted, an outside-out patch configuration was utilized to permit the external surface of the membrane to be perfused. The glass pipettes had a tip opening diameter of 0.3–0.8 μm with resistance of 2–10 megohms when filled with intracellular solution. The resistance of the seal between the pipette and cell membrane was between 5 and 10 gigaohms and was obtained by applying a small negative pressure to the interior of the pipette after its contact with the cell.

The interior of the pipette was kept at virtual ground and membrane current was measured with a current-to-voltage converter. The holding and command voltages were applied to the bath via a Ag–AgCl₀.₆₆ pellet and were measured with a separate reference microelectrode. Leakage and capacitative currents have been eliminated from the records for clarity by either subtracting averaged records with no channel openings or by adding averaged records obtained for opposite polarity pulses with the use of a digital oscilloscope (Nicolet 2090-3C) connected to a microcomputer. Records were filtered by a 6-pole Bessel filter with a cutoff frequency of 1 kHz, stored on a floppy disk, and later output to an x–y plotter for purposes of illustration.

A coverslip on which cells were grown was placed into a recording chamber mounted on a phase-contrast inverted micro-

Abbreviation: BTX, batrachotoxin. * To whom reprint requests should be addressed.

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scope. The bath was perfused and the inflow solution was cooled to control the temperature of the bath. Extracellular solution contained (in mM): Na⁺, 125; K⁺, 5.5; Ca²⁺, 1.8; Mg²⁺, 0.8; Cl⁻, 135.7; Hepes, 20; glucose, 25. The pH was adjusted to 7.3 with NaOH and the osmolarity was adjusted to 330 mosM with 20 mM sucrose. Normal intracellular solution was composed of the following (in mM): K⁺, 100, Na⁺, 1; EGTA, 20, glutamic acid, 50; Hepes, 21; sucrose, 100. Intracellular solution was made by mixing 100 mM KOH and 20 mM EGTA in water; this was followed by the addition of 20 mM Hepes (free acid) and 1 mM Na⁺ Hepes. The pH then was adjusted to 7.2 with glutamic acid and the osmolarity was adjusted to 300 mosM with ~100 mM sucrose. BTX was dissolved either in ethanol or dimethylsulfoxide to make up a 0.2 mM stock solution and was added to the intracellular or extracellular solution.

The determination of the average rate constant for closing of the channel is based on an analysis of the probability distribution for a Poisson random variable. The probability of finding closing events n in time t is given by:

\[ P(n; t) = \frac{(\nu t)^n e^{-\nu t}}{n!}, \]

where \( \nu \) is the mean rate for the event. For \( n = 0 \), the condition of not finding an event in time t, the distribution decreases to:

\[ P(0; t) = e^{-\nu t}, \]

an exponential distribution. Therefore, we plot the probability of not finding a closing for any interval t after the channel opened. This probability is:

\[ \frac{N_c}{N_{t=0}} = e^{-\nu t}, \]

in which \( N_c \) is the number open at time t and \( N_t \), as \( t \rightarrow 0 \), is the total number of channels observed. \( \nu \) is then the mean rate constant for channel closing. For multiple pathways for channel closing, the open times also will follow a single exponential with \( \nu \) being the sum of the rate constants of each closing process.

**RESULTS**

**ALTERATIONS IN THE PROPERTIES OF BTX-MODIFIED Na⁺ CHANNELS.** A comparison of selected properties of single Na⁺ channels recorded from two membrane patches, the first under normal conditions and a second that was exposed to BTX, is shown in Fig. 1A. In A, typical Na⁺ channel currents in response to membrane depolarizations are shown. A downward deflection represents an increase in inward current caused by the opening of a single Na⁺ channel. A subsequent upward deflection is caused by the closing of the channel. These currents can be associated with Na⁺ channels. First, the currents are blocked by Na⁺ channel blockers. Tetrodotoxin, a toxin which selectively blocks voltage-gated Na⁺ channels in this preparation, was applied to the external surface and caused a dose-dependent reduction in the frequency of channel openings in a series of membrane depolarizations with an E_{D50} value of 2 nM (16).

Further, single Na⁺ channel events are voltage dependent. Averages of 40–80 records show a time course similar to that measured for Na⁺ currents with whole cell voltage clamp techniques. As the membrane is depolarized to less negative potentials, the time to the peak of this average current decreases, peak amplitude increases, and the current declines with time due to inactivation. In individual records from isolated membrane patches, depolarizations to a membrane potential at which the conductance–voltage relationship reaches a maximum often show inward currents of variable amplitude. The ratio of the maximal inward current to the minimal current typically is 5, indicating that patches usually contain five channels that can open simultaneously at this potential. Depolarizations to −50 mV were utilized because this potential is at the foot of the Na⁺ conductance–voltage relationship for these cells, thus lowering the probability of opening and permitting the measurement of individual channel properties.

A histogram of the amplitudes of single channel currents at −50 mV is shown in Fig. 1B. The mean current is 1.1 pA with a standard deviation of 0.21 pA, the latter being close to the amplitude of the noise in the recording system. The variability in amplitudes follows a normal distribution. The conductance of the normal Na⁺ channel can be estimated by determining the change in the mean current amplitude at two different potentials. In one typical case, currents evoked by depolarizations from −90 mV to −46 mV and −26 mV gave mean currents of 1.3 pA and 1.1 pA, respectively. The slope conductance then is calculated to be 10 pS (8.5°C).

A Poisson plot of the lifetimes of the conducting states for the experiment represented in Fig. 1A is shown in Fig. 1C. For the closing of one open state by one or more low probability closing processes, these lifetimes should follow a single exponential distribution (see Methods And Materials). The average lifetime of the conducting state as determined by dividing the sum of the lifetimes of all observed by the number of openings observed was ~2 msec. This value is close to that of the reciprocal of the rate constant for channel closing, as determined by fitting the data to an exponential curve. The probability that a channel is open during the pulse is shown in Fig. 1D. This shows a distribution of probability vs. time that is similar to the time course of the Na⁺ current as recorded from the whole cell, because it increases with time after the onset of the pulse and later decays, apparently due to Na⁺ channel inactivation.

All three of the above parameters of single Na⁺ channels are dramatically altered when membrane patches are exposed to BTX. Results from a representative experiment in which 10 μM BTX was added to the internal solution are shown in Fig. 1E–G. The examples of membrane currents evoked by depolarizing pulses are shown in Fig. 1E. Note that the time scale is different from that in Fig. 1A. Both long- and short-duration inward currents can be seen, often in the same recording.

As in Fig. 1B–D, the distribution of current amplitude, Poisson plot of lifetime of the conducting state, and the probability of a channel being open after the start of a depolarization are plotted in Fig. 1F, G, and H, respectively. The mean amplitude was 0.79 pA. However, unlike the normal currents, the actual distribution does not match a normal distribution, and the mode is less than the mean. The most likely reason for this discrepancy is that the conducting states of Na⁺ channels are no longer homogeneous for this characteristic after exposure to BTX. A second indication for a heterogeneous population is that the distribution of open times in the Poisson plot is now better described by the sum of two exponentials (Fig. 1G). A portion of the population appears to have a slower closing rate than normal. The parameters of this exponential component were fitted by a least squares regression to the data between 60 and 150 msec. The remaining exponential component was estimated by assuming a population with a normal mean closing rate (0.5 msec⁻¹). The slower closing rate represents a reduction by a factor of about 30 compared to normal and corresponds to a mean duration of 62.5 msec.

In Fig. 1H the probability of finding a channel open during sequential time intervals is shown for the patch of membrane exposed to BTX. Three differences are apparent when comparing this measurement to the normal condition. First, this probability increased even after 20 msec after the onset of the pulse. In the normal condition, the probability was decreasing at this time. Second, this decrease in probability at later time intervals was not observed after exposure to BTX. This observation suggests that BTX-modified channels do not inactivate during the 160-msec depolarization, even though substantial inactivation...
The combination of E to BTX. One to distribution records in inward current. The combination of E to BTX. One short pulse was hyperpolarized to a holding potential of \(-90\) mV; 128 pulses then were applied to depolarize the membrane to \(-50\) mV at 4-sec intervals. Membrane current recordings during 14 of these pulses are shown in A. Inward current is downward. The time course of the depolarization \((V_t)\) is shown by the lowermost trace in A (11°C). (B) The distribution of current amplitudes for the conducting state for all of the observed Na\(^+\) channel openings over this series of pulses is given. \(X\) is the mean of the distribution and \(\sigma\) is the standard deviation. The continuous line plots a normal distribution with the parameters as given. (C) The number of channel openings that had durations greater than the times on the abscissa are plotted. The line shows a best fit to a single exponential curve with the parameters as given. \(X\) is the mean duration of the open state. The probability that a channel was open as a function of time from the beginning of the depolarizing pulse is plotted in D. The termination of the pulse is marked by the arrow. The latency and duration of each opening were first measured. The pulse duration was divided into time bins and the number of channels open during each time bin was determined and normalized to the sum total over all time bins. (E–H) The above properties were measured for a second patch of membrane in an experiment similar to that shown in A–D, except 10 \(\mu\)M BTX was added to the internal solution in the patch pipette. A holding potential of \(-90\) mV was applied to the excised membrane; 64 pulses to depolarize the membrane to \(-54\) mV then were given at 5-sec intervals. Fourteen representative examples of evoked membrane currents are shown in E. The vertical lines in each record are residual, uncompensated capacitative currents at the onset and termination of the depolarization. Note that the time scale is different from that in A (10°C). F, G, and H are the distribution of the amplitudes of the open states, the distribution of durations of the open states, and the probability of channels being open, respectively, for the membrane exposed to BTX. The solid line in G is that for the equation given and has two exponential terms (see text for further explanation). The arrow in H indicates the termination of the pulse.

Figure 1. Comparison of Na\(^+\) channel properties from membranes under normal conditions with those from membranes exposed to BTX. (A–D) An outside-out, cell-free membrane patch was produced and the membrane was dialyzed for 60 min. (E–H) The above properties were measured for a second patch of membrane in an experiment similar to that shown in A–D, except 10 \(\mu\)M BTX was added to the internal solution in the patch pipette. A holding potential of \(-90\) mV was applied to the excised membrane; 64 pulses to depolarize the membrane to \(-54\) mV then were given at 5-sec intervals. Fourteen representative examples of evoked membrane currents are shown in E. The vertical lines in each record are residual, uncompensated capacitative currents at the onset and termination of the depolarization. Note that the time scale is different from that in A (10°C). F, G, and H are the distribution of the amplitudes of the open states, the distribution of durations of the open states, and the probability of channels being open, respectively, for the membrane exposed to BTX.

Heterogeneous Population of Open States After Exposure to BTX. One interesting finding shown in Fig. 1F is that the distribution of Na\(^+\) channel current amplitudes for patches exposed to BTX is skewed to smaller currents. In the membrane current records shown in Fig. 1E, it is clear that two types of inward current steps are present. One is a larger amplitude and is of relatively short duration. A second, smaller amplitude current often occurs simultaneously and is associated with a longer duration. Because only one type of channel is present in control conditions, it is reasonable to conclude that this latter type of open state reflects that modified by BTX, whereas the large amplitude, short duration event represents an unmodified open Na\(^+\) channel. One test of this idea would be to find a positive correlation between amplitude and duration. Fig. 2 shows amplitude vs. duration scattergrams for channel openings obtained in the two experiments represented in Fig. 1. As can be seen in Fig. 2A, there is normally no correlation between the amplitude and duration of the open state of the channel. Although

occurred within 40 msec for this potential under normal conditions. The combination of a relatively high probability of finding a channel open at the end of the pulse and slow rate of channel closing produced a slowly declining "tail" after the termination of the depolarization that was not observed under normal conditions.
there is a tendency for currents with very short durations to have smaller amplitudes, this is most likely due to the limited frequency response of the recording system. During exposure to BTX, the population of Na⁺ channel openings becomes heterogeneous, as seen in Fig. 2B. Note the difference in time scale between A and B. Small amplitude currents are primarily associated with long duration events, and all of the Na⁺ channel openings with large amplitudes are associated with durations within the normal range. These observations indicate that channels having BTX-modified conducting states and those having normal conducting states coexist in the same membrane patch.

**Spontaneous Opening of BTX-Modified Na⁺ Channels.** In the presence of BTX, Na⁺ channels open spontaneously at potentials more negative than those required to open Na⁺ channels under normal conditions. If the membrane exposed to 10 μM BTX is held at −90 mV, no channel opening is observed (Fig. 3). At −80 mV and −50 mV, Na⁺ channels open spontaneously. The time between a channel opening and the next observable closing varies from 10 to 170 msec. If the exterior of the membrane is subsequently exposed to 1 μM tetrodotoxin, the transitions in membrane current are completely blocked, indicating that they are caused by the opening of Na⁺ channels that have been modified by BTX. This block is reversible after the washout of tetrodotoxin. The duration of the open state appears to be greater at −50 mV than at −80 mV because the membrane current remains inward throughout the trace in many cases.

**BTX-Modified Open States Have a Decreased Conductance.** The reduction in current amplitude for the open state modified by BTX could be caused by a reduction in the ratio of Na⁺ to K⁺ ion permeability for Na⁺ channels that occurs for BTX-modified Na⁺ current (6). To test this possibility, we recorded currents due to the opening of single Na⁺ channels in response to step depolarizations, from excised inside-out membrane patches with K⁺-free external saline solution and internal solution in which Cs⁺ had been substituted for K⁺. BTX was added to the external solution in the pipette. Although the Na⁺ to Cs⁺ permeability ratio for BTX-modified channels is near that for the Na⁺ to K⁺ permeability of normal channels (6), the membrane current for the BTX-modified conducting state of the Na⁺ channel is decreased to a similar extent under these conditions, indicating that the reduction is not caused exclusively by the decreased Na⁺ selectivity. Alternatively, the decrease in current could be caused by a reduction in the single channel conductance. The conductance for the open state of BTX-modified Na⁺ channels can be estimated by calculating the slope of the current–voltage relationship between two different potentials.

In the excised membrane exposed to 5 μM BTX, the mean current amplitudes for spontaneous openings at −70 mV and −40 mV were 0.51 pA and 0.45 pA, respectively. The conductance was calculated to be 2 pS (7°C), a value markedly lower than the normal value which varies between 10 and 12 pS. It should be noted that the standard deviation of the current amplitude is usually 0.2 pA (the rms amplitude of the noise). However, due to the large number of samples at each potential, the mean is well defined and the difference in slope conductance between the normal and toxin-modified open state appears to be significant.

**DISCUSSION**

Five points emerge from this work. (i) BTX causes a reduction in the rate of Na⁺ channel closing in the membrane potentials at the foot of the normal conductance–voltage curve, producing a prolonged lifetime of this state. A reduction in the rate of closing is caused, in part, by a shift in the activation of modified Na⁺ channels to more negative potentials (6, 7). This shift would cause the reverse rate constant for channel opening, 3α, (14), to be smaller than normal at any potential. Approximately 50% of the Na⁺ channels normally enter the inactivated state at −50 mV. Therefore, 3α, the rate constant for channels closing via inactivation (17), contributes to the mean closing rate in the control condition. The block of inactivation by BTX (6, 7) would then contribute to the decrease in the mean closing rate.

(ii) The probability of a channel being open during a maintained depolarization at −54 mV becomes constant after exposure to BTX, although channels normally inactivate during this time. The inactivation is associated with a decreased frequency of channel opening. The decreased rate of channel closings induced by BTX could explain this sustained probability. However, the mean open time for the modified open state was calculated to be 60 msec at this potential, yet the probability was constant for at least 120 msec after the time when the maximum was attained. Therefore, channels must continue to open for later times at a constant frequency.

(iii) Spontaneous opening of Na⁺ channels occurs after exposure to BTX at large negative holding potentials. This effect underlies the Na⁺-dependent depolarization and increase in Na⁺ permeability (18) of the nerve membrane by BTX. Opening of Na⁺ channels now is observed at potentials as negative as −80 mV, confirming that BTX causes Na⁺ channel activation to shift to more hyperpolarized potentials. Recently, Huang et al. (7) found that both slow and fast inactivation were inhibited by BTX. Because BTX-modified Na⁺ channels do not enter an inactivated state, they can cycle freely between open and closed states at any potential at which activation can occur.

(iv) The conductance of the BTX-modified open state of the Na⁺ channel is drastically decreased. The slope conductance measurements indicate a decrease by 80%. The ratio of the normal to modified single channel currents indicates that the conductance is decreased by only 50%, assuming linear conductance–voltage relationships and the same reversal potential.
FIG. 3. Exposure to BTX causes Na⁺ channels to open spontaneously. An outside-out excised patch of membrane was exposed to 10 μM BTX by its addition to the internal solution in the patch pipette. Recordings of membrane current were made at the three different holding potentials, which are indicated. Recordings were made at random intervals with the temporal sequence for each row being from left to right. The sequence for the series of potentials is from bottom to top. The uppermost level of current for each row of membrane current traces is nearest the zero current level. The dashed line for the records at -50 mV also marks this level of current and probably represents the condition in which all channels are closed. If the holding potential is at -80 mV or less negative, spontaneous current transitions are observed. A downward transition is an increase in inward current due to the opening of a Na⁺ channel, and an upward transition is a decrease in inward current due to the closing of a Na⁺ channel. At -50 mV, the membrane current is occasionally inward throughout the duration of the recording, indicating the persistent open state of a BTX-modified Na⁺ channel (10°C).

(Fig. 2B). Potential differences in linearity of the conductance between normal and BTX-modified open states could explain the quantitatively different estimates given by the two methods. The action of BTX to decrease single channel conductance was reported on myelinated nerve by using fluctuation analysis (19). After exposure to BTX, the maximal permeability attributed to the sum of modified and normal voltage-gated Na⁺ channels is substantially decreased (6, 7). Although one possibility for this reduction is a block of normal Na⁺ channels by BTX, the reduction can be entirely explained, on the basis of our results, by the decrease in conductance for the BTX-modified open state. All open states that exhibited a prolonged lifetime also had a decreased current amplitude. These simultaneous effects of BTX on the channel conductance and on gating kinetics are likely mediated through two allosteric sites of action, because increases in the lifetime of the open state do not always lead to reductions in this conductance (20–22) and a reduction in single channel conductance is not necessarily associated with an alteration in gating kinetics (23).

(e) The characteristics of the frequency distributions for the amplitude and duration of conducting states for Na⁺ channels exposed to BTX indicate that more than one type of open state exist. The heterogeneous population can be explained adequately by assuming that two types of open states now occur, one with normal properties and a second with a smaller mean current and decreased mean closing rate. These two states could be created in a number of ways, including the irreversible modification of a proportion of channels, reversible modification of a given channel, or alteration of any channel by BTX so that it now has both normal and modified open states. The last possibility seems less likely, because Na⁺ currents with normal characteristics are not observed in neuroblastoma cells after exposure to high concentrations of BTX (7). It has also been suggested that a portion of the population may arise from channels that are nonfunctional in the absence of modifiers similar to BTX (24).

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