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The Toxin Helothermine Affects Potassium Currents in Newborn Rat Cerebellar Granule Cells

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Abstract. Helothermine, a recently isolated toxin from the venom of the Mexican beaded lizard *Heloderma horridum horridum* was tested on K^+ currents of newborn rat cerebellar granule cells. In whole-cell voltage-clamp experiments, cerebellar granule neurons exhibited at least two different K^+ current components: a first transient component which is similar to an I_A-type current, is characterized by fast activating and inactivating kinetics and blocked by 4-aminopyridine; a second component which is characterized by noninactivating kinetics, is blocked by tetraetylammonium ions and resembles the classical delayed-rectifier current. When added to the standard external solution at concentrations ranging between 0.1 and 2 μM, helothermine reduced the pharmacologically isolated I_A-type current component in a voltage- and dose-dependent way, with a half-maximal inhibitory concentration (IC_{50}) of 0.52 μM. A comparison between control and helothermine-modified peak transient currents shows a slowdown of activation and inactivation kinetics. The delayed-rectifier component inhibition was concentration dependent (IC_{50} = 0.86 μM) but not voltage dependent. No frequency- or use-dependent block was observed on both K^+ current types. Perfusing the cells with control solution resulted in quite a complete current recovery. We conclude that helothermine acts with different affinities on two types of K^+ current present in central nervous system neurons.

Key words: Cerebellar granule neuron — *Heloderma horridum* — Helothermine — Potassium current — Patch clamp

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Introduction

Enzymatic and nonenzymatic components isolated from animal venoms have been found to affect ionic channel behaviors in different cellular preparations. Scorpion toxins affect Na^+ channel gating: α-toxins from the venoms of the scorpions *Leiurus quinquestriatus* and *Buthus eupeus* slow down channel inactivation (Catterall, 1979; Mozhayeva et al., 1980), and β-toxins from the venom of the scorpion *Centruroides sculpturatus* modulate Na^+ channel activation (Strichartz, Rando & Wang, 1987). Whole-cell and single channel experiments further enlightened the mechanisms of action of these toxins (Wang & Strichartz, 1982; Yatani et al., 1988), thus providing useful tools for studying Na^+ channel gating mechanisms. A neurotoxin phospholipase A_2 from the venom of the taipan snake *Oxyuranus scutellatus scutellatus* was shown to modify anionic currents of chick sensory neurons (Possani et al., 1992). Similarly, three animal toxins are currently extensively used to study K^+ channels: charybdotoxin (CTX) from the venom of the scorpion *Leiurus quinquestriatus,* dendrotoxin (DTX) isolated from the venom of the green Mamba snake *Dendroaspis angusticeps,* and noxiustoxin (NTX) from the venom of the scorpion *Centruroides noxius.* The peptide CTX blocks Ca^{2+}-dependent K^+ channels in quite a selective manner when tested using different preparations (Miller et al., 1985; Blatz & Magleby, 1987; Brayden & Nelson, 1992). DTX is a basic peptide affecting voltage-dependent K^+ channels in the frog node of Ranvier (Weller et al., 1985). NTX was reported (Carbone et al., 1987) to affect delayed-rectifier K^+ channels in a voltage-dependent way.

Different toxic components seem to exist in the venom of the Mexican beaded lizard *Heloderma horridum horridum*. Phospholipase A_2 with enzymatic activity (Sosa et al., 1986) and three other toxins have already been isolated (Hendon & Tu, 1981; Komori, Nikai & Sugihara, 1988; Nikai et al., 1988) from this venom. Recently, a toxin with MW 25,500 and no enzymatic activity, called Helothermine (HLTX) (the name deriving from its ability to lower body temperature when injected into mice), was purified by Mochca-Morales, Martin and Possani (1990). In this paper, we show, using the patch-clamp technique in whole-cell configuration, (Hamill et al., 1981) that HLTX affects voltage-dependent K^+ channels when tested on newborn rat cerebellar granule neurons. The choice of this primary cellular preparation was suggested by the possibility of working on a highly homogeneous (up to 98%) neuronal population of the central nervous system, as reported by Levi et al. (1984). Cerebellar neurons showed at least two K^+ current components: a fast activating and inactivating I_A-like current, 4-aminopyridine (4-AP) sensitive, and a second component, similar to the delayed-rectifier K^+ current, sensitive to tetraetylammonium ions (TEA^+). These results are in agreement with previous findings (Cull-Candy, Marshall & Ogden, 1989; Jalonen et al., 1990; Carignani et al., 1991). HLTX reduced, in a dose-dependent manner, both the I_A-like and the delayed-rectifier components, with different affinities. The main objective of the present paper is to elucidate the molecular mechanism by which HLTX induces toxic effects.

Materials and Methods

Cell Culture of Cerebellar Neurons

The experiments were performed on cerebellar granule neurons of 8–10-day-old white Wistar rats. Cerebella were mechanically dissected with a chopper and the fragments were digested in trypsin according to the procedure described by Levi et al. (1984). Dissociated neurons were plated on poly-L-lysine coated 35-mm petri dishes at a final density 2.5 to $3 \cdot 10^6$ per dish, and grown in Basal Medium Eagle (BME, Sigma, St. Louis, MO). The culture medium was supplemented with 10% fetal calf serum (GIBCO Laboratories, Eggenstein, Germany), 100 μg/ml gentamicin, 25 mM KCl and 2 mM L-glutamine. Twenty hours after plating, 50 μM cytosine arabinoside was added to prevent non-neuronal cell replication. Dishes were maintained in a 5% CO_2 incubator at 37°C. Experiments were performed 3–9 days after plating.

Electrophysiological Measurements

The composition of the standard external solution was the following (mM): 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose and 10 HEPES. Na^+ and Ca^{2+} currents were nullified by adding $3 \cdot 10^{-7}$ M tetrodotoxin (TTX) and 200 μM Cd^{2+}, to the bathing solution, respectively. The standard internal solution was (mM): 120 KCl, 5 EGTA, 0.2 $CaCl_2$, 30 glucose and 10 HEPES. The pH was adjusted to 7.3 with CsOH and NaOH for the external and the internal solutions, respectively, and osmolarity was set to 290 ± 10 mOsmol with mannitol. Electrophysiological experiments were performed by using the patch-clamp technique in whole-cell configuration (Hamill et al., 1981). Patch pipettes pulled from thick-walled borosilicate capillaries (Hilgenberg GmbH, Malsfeld, Germany) with a programmable PC-84 Sachs-Fleming puller, showed a tip resistance of 6 to 8 MΩ when filled with the standard solution. Whole-cell currents were measured with an EPC-7 amplifier (List Electronic, Darmstadt, Germany) by 200-msec-long voltage steps between −60 to 60 mV from −50 and −80 mV holding potentials (HP_s). Capacitive transient, series resistance compensation and P/4 leakage subtraction were performed on line. Acquired current data were filtered at 3 kHz. Data acquisition and analysis were performed on an Atari MEGA4 system connected to the amplifier by an analog-digital interface (Instrutech, Elmont, NY).

Helothermine Preparation

H. horridum horridum venom was obtained in the laboratories and fractionated by the chromatographic procedure described by Mochca-Morales et al. (1990). Helothermine, dissolved in the external solution, was perfused in the bath at concentrations ranging between 0.1 and 2 μM. Solution delivery was performed by using a blunt glass capillary held close to a cell, thus providing a fast solution change around the sealed cell (Carbone & Lux, 1984). The flow rate of the perfusion system was 1 ml/min. All the experiments were carried out at room temperature (20–22°C).

Results

Effect of HLTX on K^+ Current Components

Whole-cell recordings from cerebellar granule neurons, held at hyperpolarized potentials (HP ≤ −50 mV), revealed outward K^+ currents due to at least two different components: a transient, fast-activated and inactivated K^+ current, referred to as an I_A-like current, which decays to a steady-state current level in about 100 msec due to a second component, identified as a classical delayed-rectifier K^+ current. Helothermine (HLTX) externally applied at a 0.8 μM final concentration modifies K^+ currents evoked by depolarizing pulses from an HP of −80 mV, as illustrated in Fig. 1. Panel *A* shows current traces elicited by test pulses to 60 mV in a neuron held in standard solutions and after application of HLTX. The toxin reduced the peak and the steady-state current amplitudes to different ratios, as can be seen from the trace representing the blocked K^+ current. This result was obtained by digital subtraction of K^+ current in the presence of HLTX from the control K^+ current. Panels *B* and *C* show the peak and steady-state current values as functions of depolarizing pulses between −60 and 60 mV. The steady-state value was

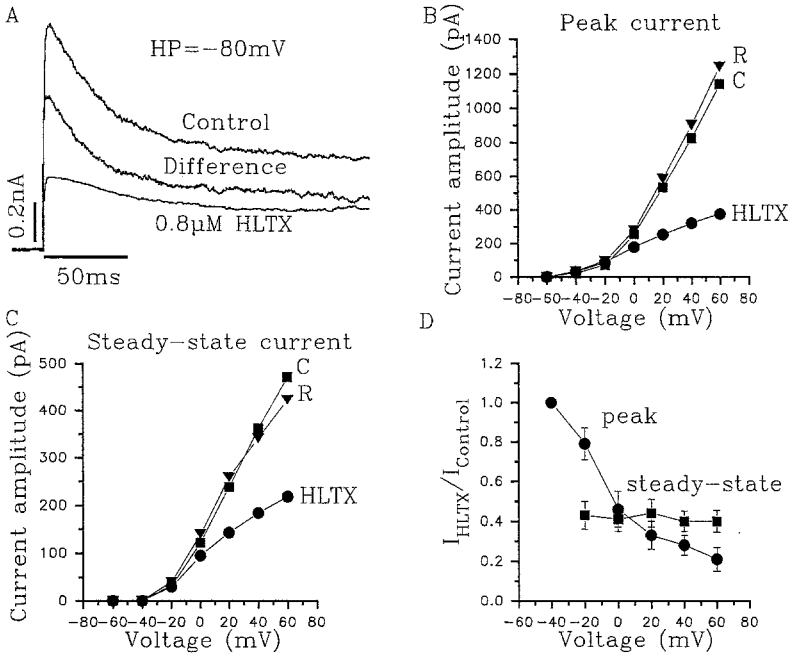

Fig. 1. HLTX induces K^+ current reduction in cerebellar granule neurons. (*A*) Current traces recorded at a 60 mV test pulse from a holding potential (*HP*) of −80 mV in control conditions and in the presence of 0.8 μM HLTX. A digitally subtracted trace, representing the K^+ current blocked by 0.8 μM HLTX, is also shown. (*B–C*) Current-voltage relationships (*I–V*) for peak and steady-state currents. The steady-state values were measured at the end of 200-msec long voltage pulses. The current amplitudes are plotted *vs.* voltage values before (■), during the application of 0.8 μM HLTX (●) and after recovery (▼). (*D*) Voltage dependence of peak (●) and steady-state (■) K^+ current inhibition. Mean values ± SEM over six cells.

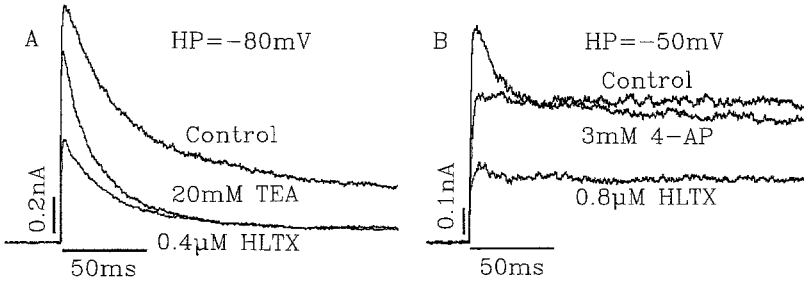

Fig. 2. HLTX affects both the transient I_A-type and the steady currents pharmacologically identified. (*A*) A neuron clamped at −80 mV shows both the transient and the late K^+ currents. Perfusion with 20 mM TEA^+ almost nullifies the delayed current. Further application of 0.4 μM reduces only the peak transient current. (*B*) A neuron clamped at −50 mV shows the two K^+ components. The transient current is blocked by 3 mM 4-AP and 50% of the steady current is blocked by 0.8 μM HLTX. Current traces evoked by a voltage pulse to 60 mV.

measured at the end of a 200-msec-long voltage pulse. The effect was completely reversed after washing HLTX out of the bath. The blocking percentage was apparently higher for the peak current values than for the steady-state current ones. In Panel *D*, the ratios between K^+ currents in the presence of HLTX and in control conditions are plotted *vs.* depolarization. The peak K^+ current inhibition by HLTX was voltage dependent, whereas no voltage dependence of the steady-state level was observed.

To observe the toxin effects on the different K^+ current components, it was necessary to perform current separation. We tested the possibility of separating the two types of components in the granule neurons by subtraction of K^+ current families evoked from different HP_s (−80 and −50 mV). But, when the cells were clamped at −50 mV, at which the I_A-like current has been reported to be virtually inactivated and just the steady component should be evoked, we noticed that, in 52 out of 67 experiments, the transient current was already present, despite the depolarized holding value. The current amplitude ratio between the two components varied considerably from cell to cell, even for the same culture.

So far, we have studied the HLTX action by use of pharmacological tools known to exert a selective block on delayed-rectifier and I_A-type currents (Cull-Candy et al., 1989). We used tetraethylammonium ions (TEA^+) and 4-aminopyridine (4-AP) at high concentrations: 20 mM TEA^+ and 3 mM 4-AP were found to be effective in blocking K^+ current components, as shown in Fig. 2. Panel *A* shows K^+ current traces evoked by test pulses to 60 mV from an HP of −80 mV in control conditions, in the presence of TEA in the external solution and after subsequent addition of HLTX to the bath. TEA^+ reduced the peak and the steady-state levels of the current by 80 and 23%, respectively. Subsequent application of HLTX at a concentration of 0.4 μM further reduced the transient current to about 55%, whereas it left the remaining steady level relatively unaffected. The toxin

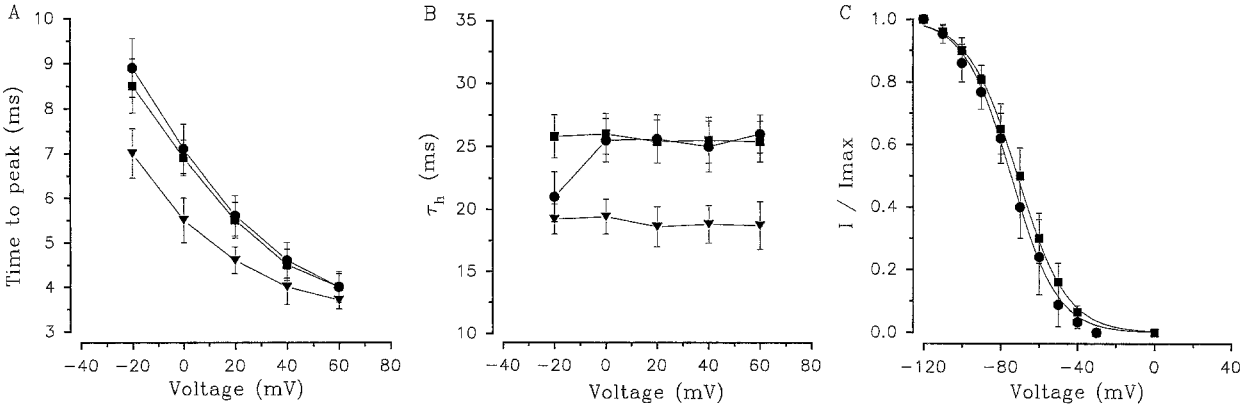

Fig. 3. HLTX effects on current activation and inactivation kinetics. (*A*) Time-to-peak for the transient K^+ current is plotted *vs.* the test potentials from HP = −80 mV in control conditions (●), in the presence of 20 mM TEA^+ (▼) and after addition of 0.4 μM HLTX (■). (*B*) Inactivation time constants (τ_h) *vs.* depolarizing pulses. The inactivation time course is described by a double exponential function in control conditions and by a single exponential function with TEA^+ (▼) and with TEA^+ plus 0.4 μM HLTX (■). In control conditions, the τ_h values (●) in the initial rapid phase of decline are plotted. (*C*) Transient K^+ current steady-state inactivation (h_∞) is not affected by HLTX. The peak current was measured during a 50-msec-long depolarizing test pulse to 50 mV, following a 500-msec-long conditioning prepulse to various potentials. Current values normalized to the value at −120 mV were fitted by a Boltzmann distribution in control conditions (●) and after adding 0.4 μM HLTX (■). Midpoint potentials are −75 and −72 mV, *k* values are 12 and 12.3, respectively. Data were averaged over three to six cells; SEM is shown.

blocking effect was entirely reversible, after a few minutes of washing, in all cells tested in these conditions (n = 35). Panel *B* shows K^+ current traces recorded at 60 mV from an HP of −50 mV and exhibiting both current components. The transient current was nullified after perfusion with 4-AP, and subsequent addition of 0.8 μM HLTX reduced the steady level to 50%. In 10 different experiments, the reductions in the steady current elicited by HLTX turned out to be equal to 43 ± 8%. In the presence of 4-AP, the delayed K^+ current inhibition was practically voltage independent (*data not shown*).

Characteristics of Toxin-affected K^+ (I_A-type) Currents

The time from the beginning of the test pulse to the peak, (i.e., time-to-peak) and the inactivation time constant (τ_h) of K^+ currents elicited by voltage steps from an HP of −80 mV were measured as parameters to illustrate the toxin effects on current activation and inactivation kinetics. As shown in Fig. 3*A*, time-to-peak, which decreases with increasing depolarizations, became faster after TEA^+ perfusion, thus suggesting a blocking mechanism on a slowly activated K^+ current. Subsequent addition of HLTX yielded time-to-peak values close to control conditions. In control conditions, the inactivation time courses, τ_h, of the transient current were found to be rather voltage independent and could be described by a sum of two exponential curves with time-constant values close to 25 and 350 msec. Instead, in the presence of either TEA^+ or TEA^+ plus HLTX, a single exponential fit was enough to describe the inactivation curve, τ_h being about 18 msec with only TEA^+ and 25 msec with TEA^+ plus HLTX. In Fig. 3*B*, the τ_h values in the initial rapid phase of current decline recorded in control conditions were compared with the τ_h values obtained in the presence of TEA^+ and after addition of HLTX to the bath. Like time-to-peak, τ_h showed a significant increase during perfusion with TEA plus HLTX. In control conditions, only at a negative test potential was a faster time constant of decay observed, as might be expected if the transient current were larger than the delayed one. The experimental data shown in Fig. 3*A–B* were obtained by holding the cells at −80 mV and by applying depolarizing pulses between −20 and 60 mV.

The steady-state inactivation process was studied by using a two-pulse protocol consisting of a 500-msec-long prepulse from −120 to −30 mV, stepped by 10 mV, followed by a 50-msec-long test pulse to 50 mV. K^+ channel availability was evaluated as the ratio I/I_{max}, namely, the ratio between each current dependent on different preconditioning potentials and the maximum current corresponding to a −120 mV preconditioning pulse. Figure 3*C* gives the I/I_{max} values *vs.* conditioning pulse potentials in control conditions and with 0.4 μM HLTX. Experimental points were fitted by a Boltzmann distribution as follows:

$$h_\infty = 1/(1 + \exp((V_m - V_{0.5})/k)) \quad (1)$$

where V_m is the preconditioning potential, $V_{0.5}$ is the midpoint potential (h_∞ = 0.5), and k is the steepness of

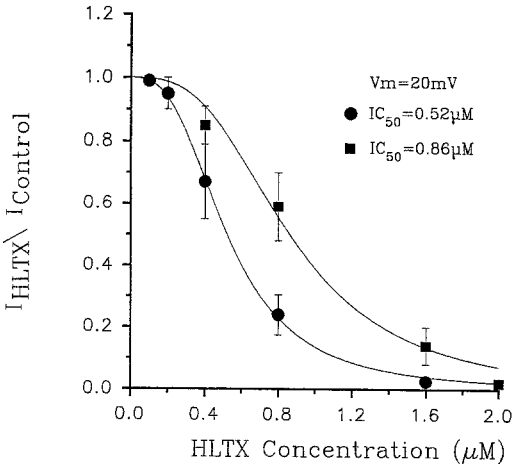

Fig. 4. Dose-response curves *vs.* toxin concentrations ranging between 0.1 and 2 μM. Experimental points are separated for transient currents (●), from HP of −80 to 20 mV in 20 mM TEA^+ external solution, and steady-state currents (■) from HP of −50 to 20 mV in 3 mM 4-AP external solution. Values of $I_{HLTX}/I_{control}$ were averaged over three to seven cells (SEM is shown) and best approximated by the equation given in the text. The half-effective doses were 0.52 and 0.86 μM for transient and late currents, respectively.

the curve. $V_{0.5}$ and k were −75 mV and 12 in control conditions, and −72 mV and 12.3 with HLTX, thus indicating a similar channel availability in both cases.

Concentration Dependence of HLTX Blocking Effects

The toxin HLTX affected K^+ conductances in a dose-dependent way. Figure 4 shows the plot of $I_{HLTX}/I_{control}$ values at 20 mV *vs.* HLTX concentrations ranging between 0.1 and 2 μM. The effect on the transient current was tested on neurons held at −80 mV and TEA^+-treated, and the effect on the delayed current was tested on neurons held at −50 mV, in the presence of 4-AP. Experimental data were fitted according to the equation:

$$I_{HLTX}/I_{control} = 1 - (C^n/(C^n + IC_{50}{}^n)) \quad (2)$$

where C is the HLTX concentration, IC_{50} is the concentration value producing a half-inhibition of current and n is the Hill coefficient. The best fit gave IC_{50} = 0.52 μM and n = 3 for the transient current, and IC_{50} = 0.86 μM and n = 3 for the delayed current, thus showing a higher sensitivity of the transient K^+ current component and indicating that three HLTX molecules must bind to a receptor in order to inhibit K^+ currents. In all the experiments, the HLTX action was fully reversible after washing the toxin out.

Time Courses and Blocking Mechanisms of HLTX

To evaluate both the time dependence of the decrease in K^+ current and recovery time, we measured currents evoked by voltage steps from an HP of −80 mV to 20 mV, at 5 sec time intervals. In Fig. 5*A*, the time courses of a HLTX block and the subsequent recovery of the transient component are evaluated in the presence of TEA^+. At 0.4 μM HLTX, steady-state inhibition was reached in less than 3 min, and recovery took about 6 min. At higher concentrations than 0.4 μM, steady-state inhibition was reached more rapidly (<2 min). An almost complete recovery was reached in a slightly longer time after washing the toxin out (≈8 min).

Many animal toxins affect ionic channels by inducing a frequency- or use-dependent blocking mechanism. To verify this possibility for HLTX, we delivered trains of six 200-msec-long pulses to 20 mV from −80 mV holding potential, at pulse intervals ranging between 5 sec and 500 msec (0.2 to 2 Hz), after a 4-min rest period. Figure 5*B* shows that, in control conditions, on a TEA-treated neuron the transient current was almost constant during each train and remained constant even when the pulse intervals were changed. HLTX (0.8 μM) reduced the current evoked by the first depolarizing pulse to about 30%; no further reduction was observed during the pulse trains and after increasing the stimulation frequency. Thus, the frequency dependence of the block was not observed. This experimental protocol was used on five other neurons. The same results were obtained for the delayed current (n = 3).

Discussion

The above results indicate that the new toxin Helothermine (HLTX), isolated from the venom of the Mexican beaded lizard *H. horridum horridum,* affects K^+ currents in cerebellar granule cells. The toxin reduced the transient and the delayed K^+ current components in a dose- and voltage-dependent way and in a dose-dependent and voltage-independent way, respectively. The half-maximal inhibitory concentrations were 0.52 μM for the peak transient current and 0.86 μM for the delayed current. The binding-site affinity on the transient channel is quite in agreement with values previously reported for toxins acting on voltage-dependent K^+ channels, the K_d values being 0.39 μM for noxiustoxin (NTX) (Carbone et al., 1987) and 0.3 μM for dendrotoxin (DTX) (Weller et al., 1985). The binding-site affinity on the delayed-rectifier K^+ channel is a little lower. Like other animal toxins, HLTX affects the gating kinetics of the channels (Yatani et al., 1988), slowing down the time-to-peak (current activation) and the inactivation time constant of the transient current. The

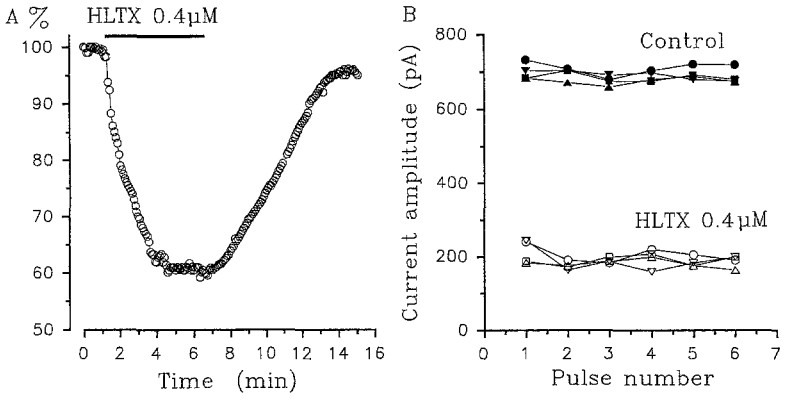

Fig. 5. Inhibition and recovery time courses of the transient current induced by the toxin. (*A*) Block rising was measured by applying successive depolarizing pulses to 20 mV from HP of −80 mV at 5 sec time intervals. HLTX (0.4 μM) was applied for the bar duration. (*B*) Use- or frequency-dependent HLTX blocking effect on the transient K^+ current. Trains of test pulses from HP of −80 mV to 20 mV at different time intervals were applied. Filled symbols indicate control values and open symbols indicate recordings in the presence of 0.8 μM HLTX. Stimulation frequencies were: 0.2 Hz (circle), 0.5 Hz (downward triangle), 1 Hz (square) and 2 Hz (upward triangle).

blocking effect is reversed after washing HLTX out of the bath solution. No frequency- or use-dependent action of the toxin occurred on both the transient current and the delayed one. A comparison of the NTX, DTX and HLTX effects on K^+ currents shows that the main differences concern voltage sensitivities and use dependences. The K^+ channel blockage by NTX or DTX at high concentrations exhibits close similarities to that of 4-AP in the voltage-dependent removal of the block and in the frequency dependence (Yeh et al., 1976; Weller et al., 1985; Carbone et al., 1987). The voltage dependence of the I_A-type current block induced by HLTX could be explained as being due to a negatively charge toxin acting on the extracellular side of the channel protein. In fact, HLTX has an acidic isoelectric point (pI = 6.8) and contains 57 negatively charged amino acids that, together with a high molecular weight, make unlikely its solubilization into the hydrophilic core of the plasma membrane. Other relatively large peptides, like NTX and, in general, scorpion and coelenterate toxins, must be applied to the external membrane surface to affect the channels (Hille, 1992). Concerning the delayed K^+ current, it was difficult to record without activating the transient component, and this may explain the difficulties with verifying the toxin effects on this stationary current. Furthermore, the diverse dose and voltage dependences of the two K^+ current components due to HLTX cannot be easily predicted by a simple model. Thus, further investigations at the single channel level will allow us to separate different channels to obtain new information on the HLTX mechanism of action on K^+ channel gatings. Compared with whole-cell measurements (which show two K^+ current components), single channel experiments indicated the presence of at least three types of K^+ channels (Jalonen et al., 1990) different from a Ca^{2+}-dependent K^+ channel (Fagni, Bossu & Bockaert, 1991). This suggests that only a detailed analysis of single channel fluctuations will elucidate a specific affinity of the toxin for a K^+ channel type.

However, other aspects are worth considering. Animals injected with 75 μg of toxin per 20 g of body weight died after showing some of the following symptoms: lethargy, partial paralysis of rear limbs, diarrhea, and lowering of body temperature. If intoxicated animals are touched in the dorsal parts of their bodies, sporadic tonic convulsions occur. Furthermore, electrophysiological experiments conducted on squid axon membranes, chick dorsal root ganglion neurons and newborn rat heart cells indicated that HLTX does not affect Na^+, K^+ and Ca^{2+} permeabilities (Mochca-Morales et al., 1990). Our findings indicate that the central toxicity and the convulsive action of HLTX may be ascribed to a reduction in the transient K^+ conductance in cerebellum granule cells, but we do not know which of the above-mentioned symptoms may be attributed to the delayed K^+ current block. More interdisciplinary studies will certainly help clarify the complexity of the toxin effects.

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