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A new class of neurotoxin from wasp venom slows inactivation of sodium current

Yoshinori Sahara, Masamichi Gotoh,[1] Katsuhiro Konno,[2] Akiko Miwa,[3] Hiroshi Tsubokawa,[4] Hugh P. C. Robinson[5] and Nobufumi Kawai[4]

Department of Physiology, Faculty of Dentistry, Tokyo Medical and Dental University, Tokyo 113-8549, Japan
[1]Department of Endodontics, Faculty of Dentistry, Tokyo Medical and Dental University, Tokyo 113-8549, Japan
[2]Institute of Biosciences of Rio Claro, Sao Paulo State University, Rio Claro, Brazil
[3]Department of Neurobiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183-8536, Japan
[4]Department of Physiology, Jichi Medical School, Tochigi 329-0498, Japan
[5]Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK

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Abstract

The effects of α-pompilidotoxin (α-PMTX), a new neurotoxin isolated from the venom of a solitary wasp, were studied on the neuromuscular synapses in lobster walking leg and the rat trigeminal ganglion (TG) neurons. Paired intracellular recordings from the presynaptic axon terminals and the innervating lobster leg muscles revealed that α-PMTX induced long bursts of action potentials in the presynaptic axon, which resulted in facilitated excitatory and inhibitory synaptic transmission. The action of α-PMTX was distinct from that of other known facilitatory presynaptic toxins, including sea anemone toxins and α-scorpion toxins, which modify the fast inactivation of Na^+ current. We further characterized the action of α-PMTX on Na^+ channels by whole-cell recordings from rat trigeminal neurons. We found that α-PMTX slowed the Na^+ channels inactivation process without changing the peak current–voltage relationship or the activation time course of tetrodotoxin (TTX)-sensitive Na^+ currents, and that α-PMTX had voltage-dependent effects on the rate of recovery from Na^+ current inactivation and deactivating tail currents. The results suggest that α-PMTX slows or blocks conformational changes required for fast inactivation of the Na^+ channels on the extracellular surface. The simple structure of α-PMTX, consisting of 13 amino acids, would be advantageous for understanding the functional architecture of Na^+ channel protein.

Introduction

Voltage-sensitive Na^+ channels are responsible for generating action potentials in most excitable tissues. Membrane depolarization causes a voltage-dependent conformational change that increases the permeability to sodium ions. This is followed by inactivation, wherein the channel closes and the permeability to sodium ions is shut off (Hille, 1992). The proteins that comprise the sodium channel have been purified from a variety of tissues and species, and cDNA clones encoding the channels have been isolated from a variety of tissues (for reviews, see Catterall, 1988, 1995; Trimmer & Agnew, 1989; Stühmer & Parekh, 1992). Tissue-specific differences in the effects of Na^+ channel-specific neurotoxins indicate the existence of different subtypes (for reviews, see Catterall, 1980, 1995; Strichartz *et al*., 1987).

During a search for novel neuroactive substances from biological sources, we isolated a peptide toxin which was named α-pompilidotoxin (α-PMTX), in the venom of a solitary wasp, *Anopolis samariensis* (Konno *et al*., 1997, 1998). α-PMTX, a 13-amino acid peptide, greatly facilitates both excitatory and inhibitory synaptic transmission in the lobster neuromuscular synapse (Konno *et al*., 1997), as well as disrupts synchronous firing in rat cortical neurons (Harsch *et al*., 1998). Because the resting conductance of the postsynaptic membrane is not affected, α-PMTX appears to act mainly on the presynaptic membrane. However, effects of α-PMTX were clearly different from those of known presynaptic toxins, e.g. carybdotoxin or apamin, which affect Ca^{2+}-activated K^+ channels. Also, α-PMTX did not affect the spontaneous miniature potentials, indicating that the toxin has a distinct action from that of α-latrotoxin (Konno *et al*., 1997). Although above results suggest that α-PMTX acts on the presynaptic membrane, more direct evidence is necessary to clarify the ionic mechanism underlying the synaptic facilitation. We therefore employed intracellular recordings from the presynaptic axon in the neuromuscular synapse of lobster walking leg. The preparation offers an advantage for recording both presynaptic and postsynaptic potentials simultaneously (Kawai & Niwa, 1980; Miwa *et al*., 1993). We also carried out whole-cell voltage-clamp recordings in rat trigeminal ganglion (TG) neurons and found that α-PMTX has the unique action of slowing Na^+ current inactivation. By chemical modification of α-PMTX, we found that certain amino acids are crucial to its effect on inactivation of Na^+ current.

Materials and methods

Lobster walking leg preparations

Neuromuscular preparations of the walking leg of lobster (*Palinurus japonicus*) were employed as described previously (Kawai & Niwa, 1980; Miwa *et al*., 1993). The excitatory and inhibitory axons

Correspondence: Professor N. Kawai, as above.
E-mail: kawainob@jichi.ac.jp

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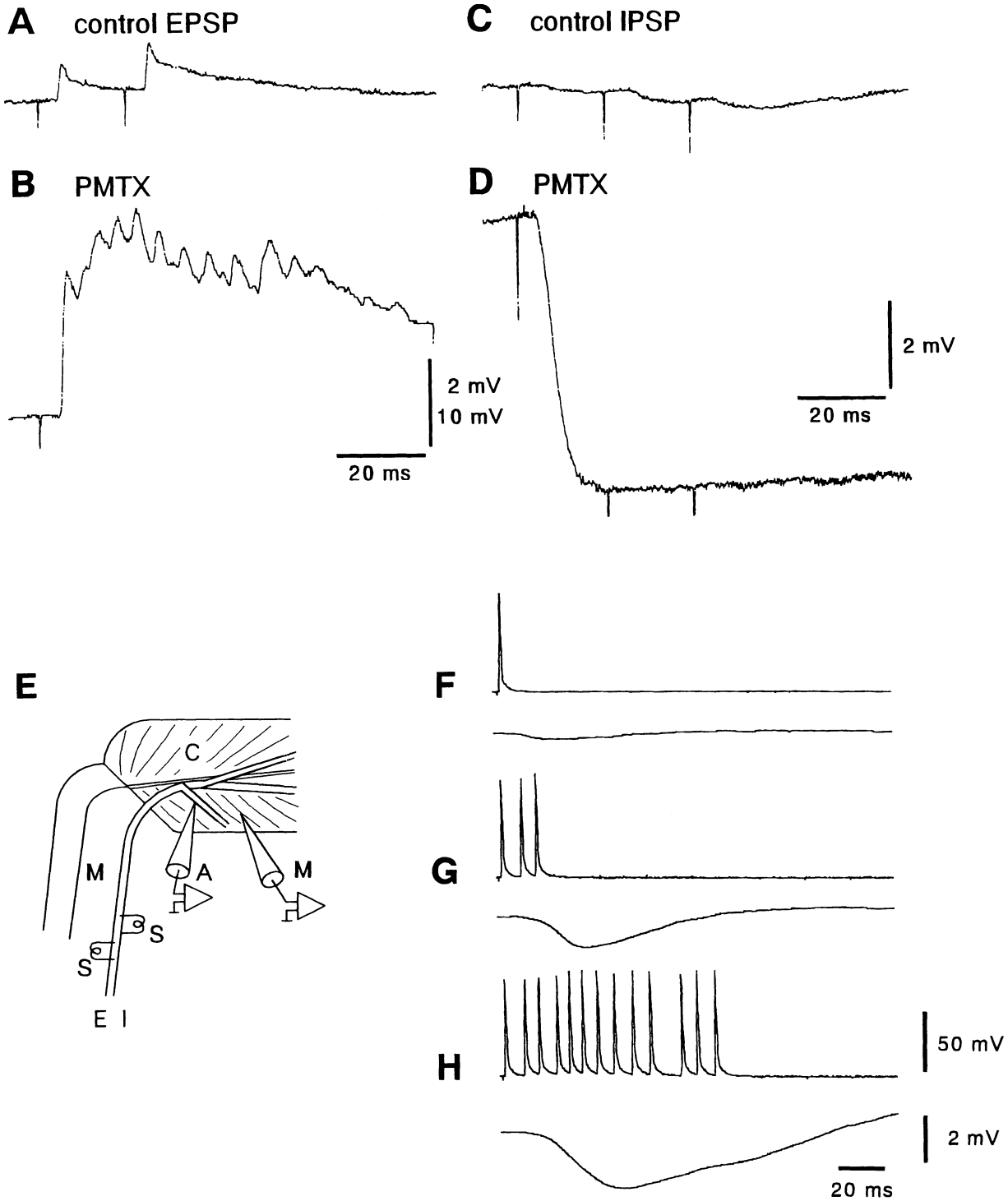

FIG. 1. Effect of α-PMTX on the neuromuscular synapse in the lobster walking leg. (A) EPSPs evoked in the stretcher muscle by paired stimulation of the excitatory nerve at an interval of 20 ms in normal solution. (B) Five minutes after application of 10 μM α-PMTX. (C) Control IPSPs evoked by three successive stimuli to the inhibitory nerve at intervals of 20 ms. (D) Five minutes after application of 10 μM α-PMTX. The resting membrane potential was –65 mV. The voltage scale is 2 mV for A and 10 mV for D. (E) Arrangement of electrodes in the lobster walking leg preparation. C, carpopodite; M, meropodite; E, excitatory nerve; I, inhibitory nerve; S, stimulating electrode; A, recording electrode for the presynaptic inhibitory axon; M, recording electrode for the stretcher muscle. (F–H) Upper traces are intracellular records from the inhibitory axon. Lower traces are simultaneously recorded IPSPs in the stretcher muscle. (F) control; (G) 5 min; and (H) 6 min after application of α-PMTX (10 μM). Single stimulation induced repetitive firing in the inhibitory axon and summating IPSPs in the muscle. The resting membrane potential was –65 mV (axon) and –70 mV (muscle), respectively.

innervating the stretcher muscle were isolated at the meropodite and stimulated independently. Both excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) were recorded from the stretcher muscle. Intracellular recordings were made from the inhibitory nerve near the nerve terminal using microelectrodes filled with 4 M K acetate (10–20 MΩ). The normal bath solution consisted of (in mM): NaCl, 468; KCl, 10; $CaCl_2$, 20; $MgCl_2$, 8; Tris buffer, 2; adjusted to pH 7.4.

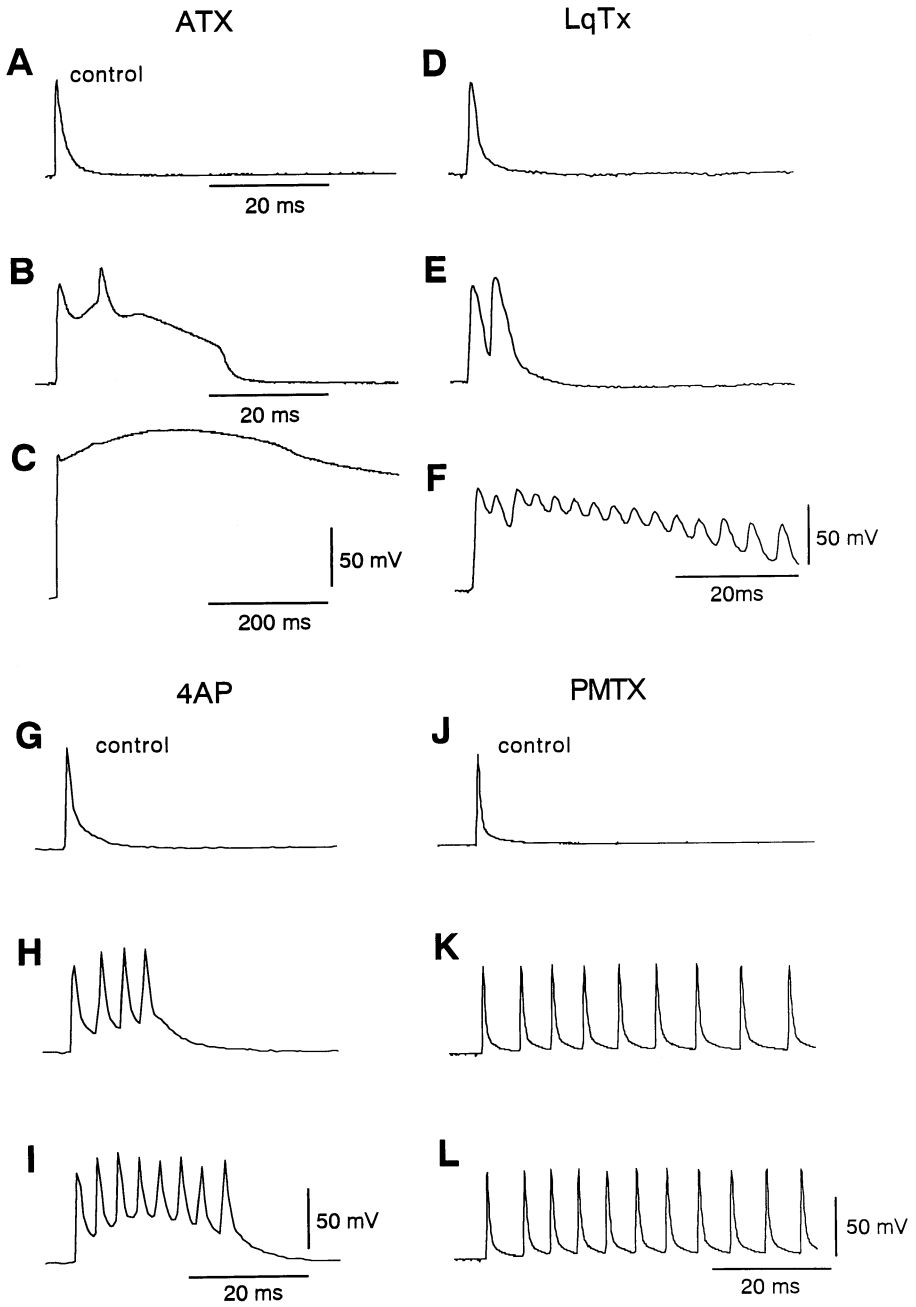

FIG. 2. Comparison of the effects of various neurotoxins and 4-AP on lobster axon action potentials. In each set of records, the top traces (A, D, G and J) are controls. (B) Three minutes, and (C) 5 min after application of ATXII (100 nM). (E) Ten minutes, and (F) 11 min after application of α-scorpion toxin (LqTx Ia, 100 nM); (H) 1 min, and (I) 5 min after application of 4-aminopyridine (5 mM); (K) 5 min, and (L) 8 min after application of α-PMTX (10 μM). The resting membrane potential was –65 mV (A–C), –60 mV (D–F), –63 mV (G and H), and –65 mV (J–L).

Rat trigeminal ganglion neurons

Trigeminal ganglia were isolated from neonatal (3–6 days old) Wistar rats as described previously (Sahara *et al*., 1997). Rats were anaesthetized, and dissected ganglia were incubated in Hank's solution (Gibco, Gaithersberg, MD, USA) with papain (20 U/mL, Worthington Biochemical, Freehold, NJ, USA) at 37–38 °C for 15 min. Cells were dissociated by trituration using a sterile Pasteur pipette, and subsequently plated onto poly-L-lysine-pretreated 35-mm culture dishes at a density of 2×10^3 cells per plate. The plating medium consisted of Leibovitz's L-15 solution (Gibco), 10% foetal calf serum, penicillin–streptomycin (20 U/mL), 26 mM $NaHCO_3$ and 30 mM glucose. The cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37–38 °C, and used for recording between 6 and 12 h after plating. The hippocampal cell culture methods were identical to those described previously (Sahara & Westbrook, 1993).

Whole-cell recording was carried out with an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA, USA) at room temperature. Pipettes for whole-cell recording contained (in mM): CsF, 110; NaF, 10; CsCl, 40; $MgCl_2$, 2; ethylene diamine tetraacetic acid (EDTA), 11; *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulphonic acid] (HEPES), 10; pH 7.3; osmolarity was adjusted to 310 mOsm. The extracellular solution contained (in mM): NaCl,

25–50; KCl, 1; $MgCl_2$, 1; HEPES, 10; glucose, 10; tetraethylammonium (TEA) chloride, 110; pH 7.2; osmolarity 325 mOsm. An array of six quartz-glass tubes (400 µm in diameter, Polymicro Tech, Phoenix, AZ, USA) was used for changing solutions. Each flow tube was connected to a gravity-fed reservoir and controlled by a three-way solenoid valve. All records were filtered at 5 kHz (48 dB/oct, NF System, Tokyo, Japan) and digitized using pClamp (Axon Instruments, sampling rate 10–20 kHz) on a computer (Compaq Prolinear 4/33i) with a TL-1 DMA digital interface. The digitized records were analysed using Axograph (Axon Instruments).

α-PMTX was synthesized by an automated solid-phase peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan) based on the Fmoc-strategy (Konno *et al.*, 1998). Sea anemone toxin (ATXII) was purchased from Calbiochem (La Jolla, CA, USA), scorpion toxin (LqTx-Iα) was purchased from Latoxan (Rosass, France) and tetrodotoxin (TTX) from Wako Pure Chemical (Osaka, Japan).

Results

α-PMTX modifies action potential generation in the presynaptic axon on the lobster neuromuscular synapse

In the stretcher muscle of the lobster walking leg, EPSPs and IPSPs were evoked by stimulating single excitatory and inhibitory axons, respectively (Fig. 1A and C). When α-PMTX (10 µM) was applied to the neuromuscular synapse, both the EPSP and IPSP were greatly potentiated. Stimulation of the excitatory nerve caused a large oscillatory depolarization showing summated EPSPs (Fig. 1B). In the same muscle fibre, stimulation of the inhibitory axon induced a prolonged plateau hyperpolarization presumably at the equilibrium potential for the IPSP (Fig. 1D). α-PMTX had no effect on the resting conductance of the postsynaptic membrane, nor does it affect the excitability of the muscle membrane as tested by intracellularly applied current pulses. These results imply that the toxin acts by modifying action potential generation in the presynaptic axon. In order to test this, we carried out paired intracellular recording from the presynaptic axon near the nerve terminals and from innervating the stretcher muscle (Fig. 1E). We used inhibitory rather than excitatory axons, to avoid possible artefacts due to muscle contraction when stimulating the excitatory axon. In the control, a single stimulation of the axon elicited an action potential which produced a small IPSP (Fig. 1F). After application of α-PMTX, single stimulation produced repetitive action potentials resulting in summated IPSPs in the muscle (Fig. 1G). This effect increased with time (Fig. 1H), presumably reflecting gradual diffusion of the toxin to the tissue. When the preparation was extensively washed with normal solution shortly after toxin application, the effect was reversible and recovered to the control response. However, after the preparation was soaked in the toxin solution for several minutes, long bursts of action potentials were repeatedly induced by single stimulation. The shape of the action potentials did not change significantly.

Comparison of α-PMTX with other neurotoxins at the lobster axon

We compared the effects of α-PMTX with those of other peptide toxins which have facilitatory actions. When sea anemone toxin (ATXII) from *Anemonia sulcata,* which is known to block Na^+ current inactivation (Bergman *et al.*, 1976; Romey *et al.*, 1976; for review, see Catterall, 1980; Alsen, 1983; Strichartz *et al.*, 1987), was applied to the lobster axon, the action potential was greatly prolonged and this was sometimes accompanied by multiple spike potentials (Fig. 2B). Later, the action potential developed a long-lasting plateau phase (Fig. 2C). Some scorpion toxins are known to block Na^+ current inactivation similarly to the sea anemone toxin (for review, see Catterall, 1980; Strichartz *et al.*, 1987; Eitan *et al.*, 1990). The effect of LqTx-Iα (100 nM) derived from scorpion (*Leiurus quinquestratus*) venom resembled that of the sea anemone toxin in that it caused repetitive spike potentials in the lobster axon (Fig. 2E and F). 4-Aminopyridine (4-AP, 5 mM), which blocks transient outward and delayed rectifier K^+ channels, caused bursting spike potentials (Fig. 2H and I). However, the repetitive action potentials were superimposed on a long-lasting depolarization of the membrane potential. The mode of action of α-PMTX is unique in that the repetitive spike potentials always start from the baseline resting potential (Fig. 2K and L). These findings suggest that α-PMTX may act by influencing the activation or inactivation of Na^+ channels, but in a fundamentally different manner from previously known neurotoxins.

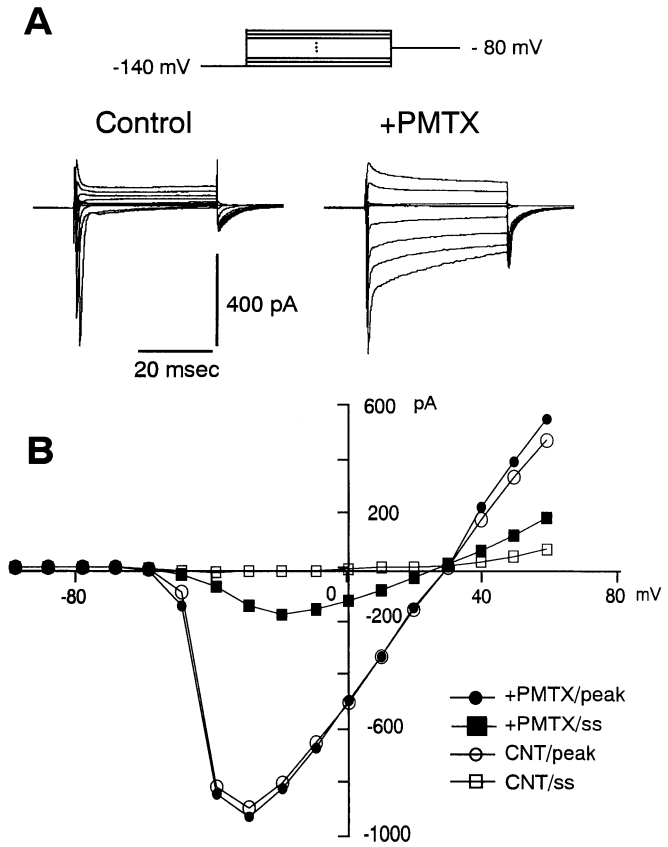

FIG. 3. Effects of α-PMTX on Na^+ currents in rat TG neurons. (A) Superimposed TTX-sensitive Na^+ current traces activated by depolarization in 20-mV increments from –140 mV to +60 mV before and 1 min after application of 10 µM α-PMTX. Na^+ currents were leak subtracted (P/4 protocol) and the membrane potential was held at –140 mV. (B) Current–voltage relationships of the peak Na^+ current (peak, circles) and the steady-state Na^+ current (ss, squares) measured at 30 ms from the beginning of the step voltage, in the absence (CNT, open symbols) and presence (+PMTX, filled symbols) of α-PMTX.

α-PMTX slowed Na^+ current inactivation in trigeminal ganglion neurons

Because the fine structure of the presynaptic axons near the nerve terminals is not suited for voltage clamping, we employed whole-cell recordings from acutely dissociated TG neurons to study the mode of action of α-PMTX on the Na^+ currents. Families of superimposed

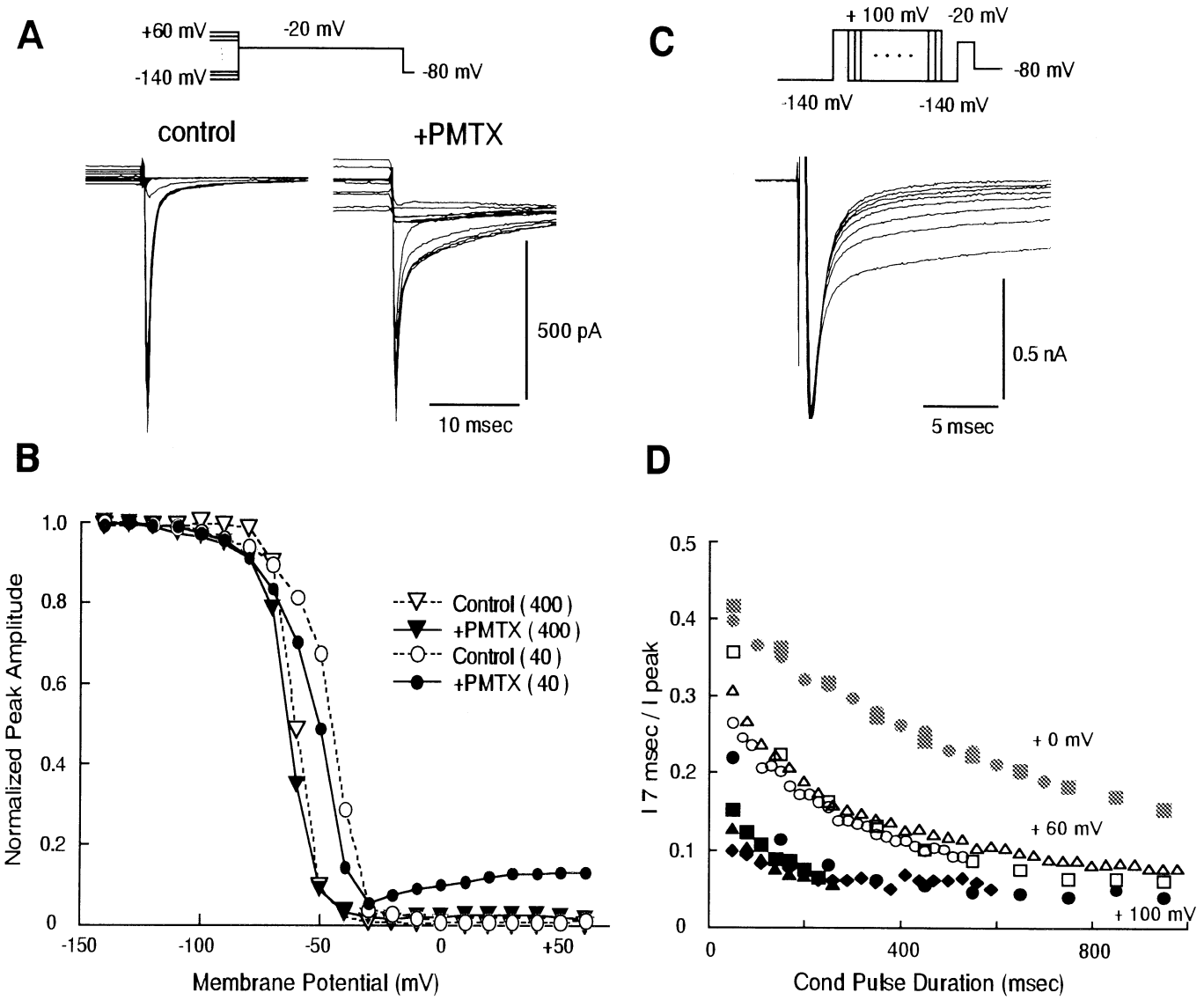

FIG. 4. Effects of α-PMTX on Na^+ channel inactivation and voltage-dependent loss of the toxin action. (A) Na^+ current traces at various conditioning prepulses of 40 ms before (control) and after (+PMTX) α-PMTX treatment. (B) Relative Na^+ permeability measured with a conditioning pulse for 40 ms (circles) or 400 ms (triangles) at various membrane potentials. Peak Na^+ conductance was calculated before (open circles) and after treatment (closed circles) with α-PMTX. The relative peak Na^+ current amplitude was normalized by the maximum peak Na^+ current amplitude in each panel and plotted against the conditioning prepulse potential. (C) The acceleration of inactivation with longer conditioning pulses. The rate of loss of toxin action at +100 mV was determined by stepping to a conditioning pulse for 50–1450 ms, returning to –140 mV for 20 ms to recover from inactivation, then eliciting Na^+ current with a 40 ms test pulse to –20 mV. (D) The ratio of current 7 ms after the peak ($I_{7\,ms}$) to the current at the peak (I_{peak}) was plotted as a function of time during depolarization pulses of +100 mV (shadow symbols), +60 mV (open symbols) and 0 mV (filled symbols). Data from two to four cells with different symbols in each depolarization pulse are superimposed.

Na^+ current traces produced by steps to voltages between –140 mV and +60 mV are shown in Fig. 3A. The obvious effect of α-PMTX (10 μM) on Na^+ currents is a prolongation of the decline of Na^+ currents. The current–voltage relationships show that the steady-state Na^+ current, measured at 30 ms from the beginning of the voltage step, is dramatically increased by α-PMTX in a voltage-dependent manner (Fig. 3B, closed squares). The steady-state current (I_{ss}) reaches its maximum value near –20 mV, whereas the peak current (I_{peak}) reaches its maximum value near –30 mV. The I_{ss}/I_{peak} percentage ratios at –20 mV were 0.9 ± 0.2 (mean ± SEM, $n = 10$) in the control and 14.8 ± 2.4 ($n = 5$) in the presence of α-PMTX. Neither the peak amplitude nor the rising phase of the Na^+ currents was affected by α-PMTX, suggesting that the toxin interferes selectively with the Na^+ channel inactivation processes without affecting their activation. α-PMTX was only active when applied externally. Inclusion of α-PMTX into the whole-cell pipette elicited no effects on the Na^+ currents. α-PMTX did not shift the reversal potential of the Na^+ currents, indicating that the ion selectivity of the Na^+ channel remained almost unchanged.

A two-pulse protocol was used in which a 40-ms prepulse of various conditioning potentials was followed by a test pulse at –20 mV (Fig. 4A). Normalized peak Na^+ currents were plotted against the conditioning prepulse potentials (Fig. 4B). In the control, the relative amplitudes of the peak Na^+ currents declined monotonically with increasing positive prepulse potentials and most of the Na^+ channels were inactivated (Fig. 4B, open circles). In contrast, in the presence of α-PMTX, a significant fraction of the Na^+ channels was still activated by a prepulse of –30 mV. This fraction increased

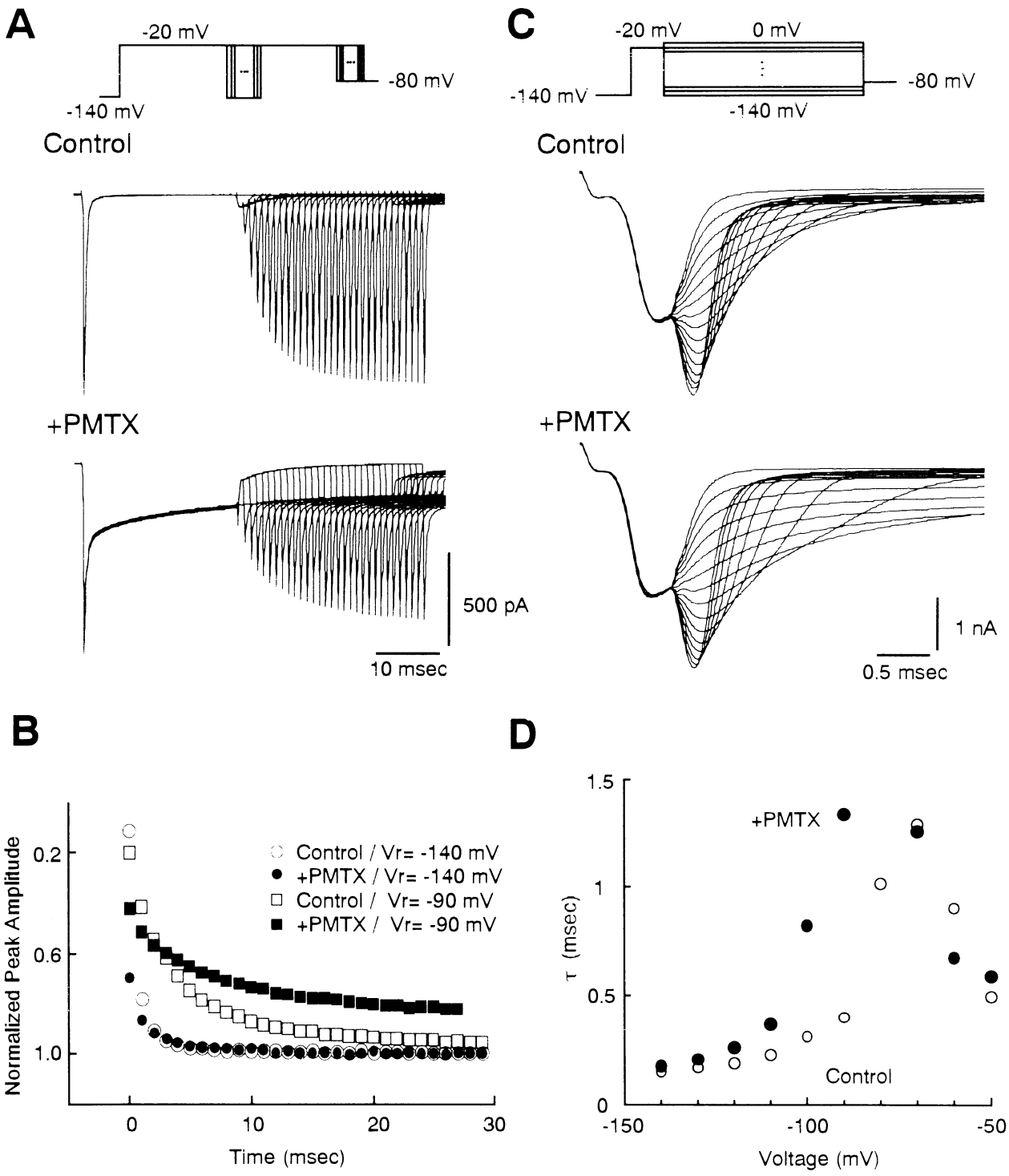

FIG. 5. Effects of α-PMTX on the rate of recovery from inactivation and deactivating tail currents. (A) Recovery from inactivation at different membrane potentials. The cell was held at –140 mV and pulsed twice to –20 mV for 40 ms with a gradually increasing interval between the two pulses at –140 mV or –90 mV (recovery potential, V_r). (B) The horizontal axis is the duration of the interval between two pulses, and the vertical axis is the extent of recovery. The extent of recovery was normalized to the peak Na^+ current in the first pulse. (C) Deactivating tail currents with and without α-PMTX. Na^+ current was activated by a 0.5-ms pulse to –20 mV and then repolarized from –140 mV to 0 mV in 10-mV increments to observe channel deactivation before significant macroscopic inactivation occurred. (D) The vertical axis is the tail current deactivation rate, and the horizontal axis is the voltage. Time constants are determined by single exponential fits to 20–80% of the decay. Open symbols represent the time constants from cells before application of α-PMTX, and closed symbols represent those after the application of α-PMTX.

continuously with depolarizing prepulse potentials until it reached an apparent plateau value, although there was a slight reduction in the steepness of the voltage dependence of inactivation (Fig. 4B, filled circles). However, when we used 400-ms prepulses to measure the voltage dependence of steady-state inactivation, the effects of α-PMTX were almost abolished (Fig. 4B, filled triangles).

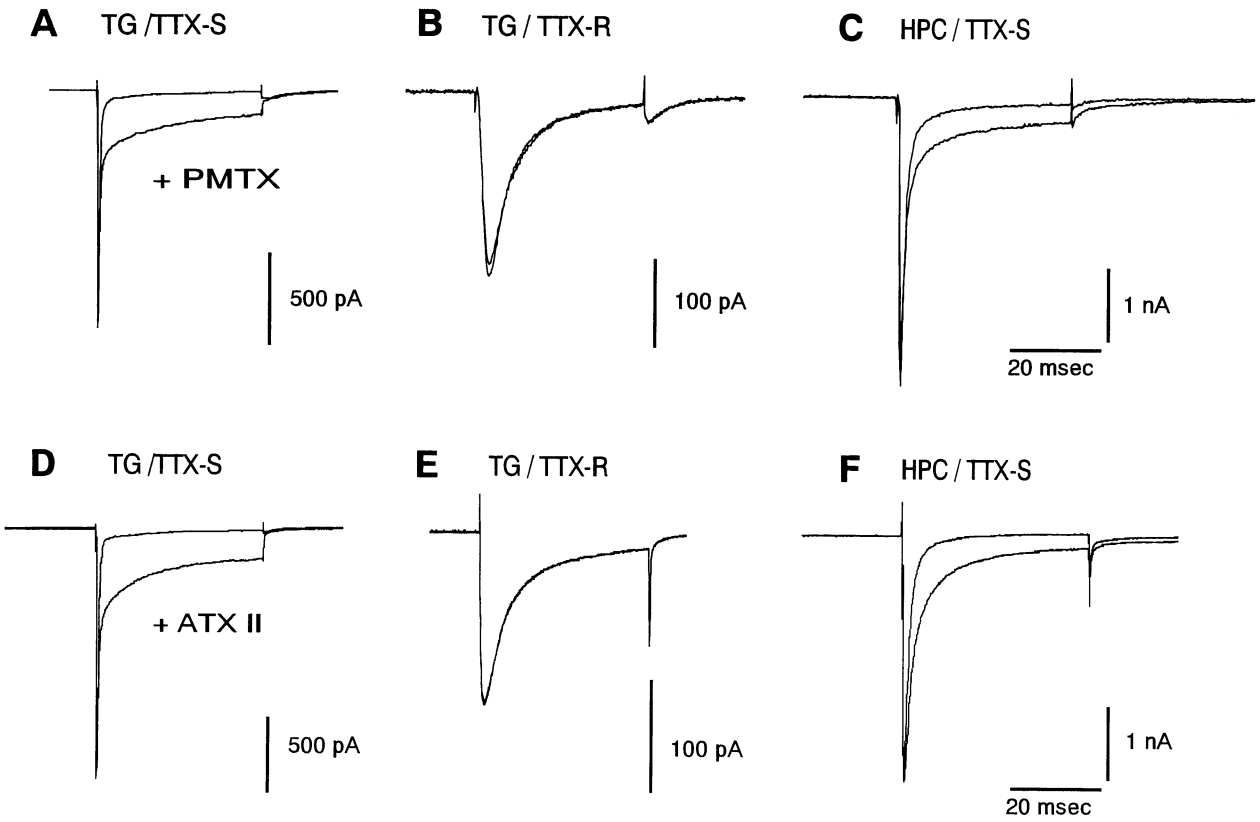

FIG. 6. Comparison of the effects of α-PMTX with those of ATXII on various Na^+ currents. The records represent superimposed traces before and after application of α-PMTX (10 μM) for 1 min. (A) TTX-sensitive Na^+ currents from dissociated TG neurons (TG/TTX-S). (B) TTX-resistant Na^+ currents from TG neurons (TG/TTX-R). (C) TTX-sensitive Na^+ currents from cultured hippocampal neurons (HPC/TTX-S). (D–F) Superimposed traces from TTX-sensitive and TTX-resistant Na^+ currents of dissociated TG neurons and hippocampal neurons before and after ATXII (1 μM) treatment for 1 min. The external medium contained TTX (1 μM) during recording of TTX-resistant Na^+ currents. TTX-sensitive Na^+ currents were activated by depolarization from –140 mV to –20 mV, and TTX-resistant Na^+ currents were activated by depolarization from –100 mV to –10 mV. Na^+ currents were leak subtracted (P/4 protocol).

α-PMTX has voltage-dependent actions on Na^+ currents

It has been known that the binding of neurotoxins, which slow Na^+ current inactivation, is inhibited by depolarization due to acceleration of toxin dissociation (Catterall, 1979; Strichartz & Wang, 1986; Rogers *et al*., 1996). We therefore tested whether the loss of α-PMTX action was voltage dependent. As shown in the protocol in Fig. 4C, TG neurons were held at –140 mV to stimulate maximum toxin binding, then depolarized to +100 mV for intervals from 50 to 1450 ms to reduce toxin dissociation, repolarized briefly to –140 mV to reverse channel inactivation and depolarized to –20 mV for measurement of the Na^+ currents (Fig. 4C). Prolonged conditioning pulses for a few hundred ms reduced the effects of α-PMTX. We then compared the time course of the loss of toxin action at the different depolarizing potentials, +100 mV, +60 mV and 0 mV. By calculating the ratio of $I_{7\,ms}/I_{peak}$ as a function of the conditioning pulse duration, the time course of loss of toxin action was plotted. As shown in Fig. 4D, the rate of decrease in toxin action was voltage dependent, with more rapid decrease during stronger depolarization.

Rate of recovery from inactivation by α-PMTX

We next studied the effects of α-PMTX on the rate of recovery from inactivation. A two-pulse protocol with various duration of the interpulse intervals was employed (Fig. 5A). In the control, the rate of recovery of the Na^+ currents was described by the sum of two exponential functions, a fast component (τ_f) with a time constant of 4.59 ms and a slow component (τ_s) of 29.81 ms (Fig. 5B, open circles). In agreement with the data from other laboratories (Armstrong & Bezanilla, 1977; Kuo & Bean, 1994), recovery from Na^+ current inactivation occurred faster at a more negative recovery voltage ($V_r = -140$ mV) than at $V_r = -80$ mV. At $V_r = -140$ mV, the fast component (τ_f) had a time constant of 0.61 ms and the slow component (τ_s) of 5.08 ms (Fig. 5B, open squares). After application of α-PMTX, although the rate of recovery was almost unchanged at $V_r = -140$ mV (Fig. 5B, filled circles), it was considerably delayed at $V_r = -80$ mV. The rate of recovery of Na^+ currents consisted of a τ_F of 1.28 ms and a τ_s of 65.32 ms (Fig. 5B, filled squares). The recovery from inactivation is known to be tightly coupled with channel deactivation, analogous to the coupling between Na^+ channel activation and the development of inactivation (Kuo & Bean, 1994). Therefore, we compared the kinetics of recovery from inactivation (for channels that have been inactivated) with that of channel deactivation (for channels that have not been inactivated) in the presence of α-PMTX. Tail currents following a brief activating pulse decayed with a time course that could be well approximated by a single exponential decay, with a strong voltage-dependent time constant (Fig. 5C and D). Recovery from inactivation was much slower than the rate of deactivation. α-PMTX did not change the rate

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*		Relative potency
α-PMTX	R	I	K	I	G	L	F	D	Q	L	S	K	L	NH_2	1.0
β-PMTX	R	I	K	I	G	L	F	D	Q	L	S	**R**	L	NH_2	5.0
PMTX-OH	R	I	K	I	G	L	F	D	Q	L	S	K	L	**OH**	0.0
Y7F	R	I	K	I	G	L	**Y**	D	Q	L	S	K	L	NH_2	0.0
F2I	R	**F**	K	I	G	L	F	D	Q	L	S	K	L	NH_2	0.0
PMTX(1-9)	R	I	K	I	G	L	F	D	Q	NH_2					0.0
PMTX(6-13)						L	F	D	Q	L	S	K	L	NH_2	0.0

FIG. 7. Structure–activity relationships of synthetic analogues of α-PMTX. The threshold concentration of the analogue for inducing repetitive action potentials in the lobster axon was estimated. The relative potency of the analogue was determined by comparing the threshold concentration with that of α-PMTX. When the analogue was ineffective with the concentration of 10 mM the relative potency indicates 0.

of deactivation at more negative potentials, but slowed around the resting potential (Fig. 5D, closed circles).

α-PMTX has no effect on TTX-resistant Na^+ currents

Sensory ganglion neurons such as those in TG and dorsal root ganglion are known to express TTX-resistant Na^+ channels (Roy & Narahashi, 1992; Elliott & Elliott, 1993; Akopian *et al*., 1996). The TTX-resistant Na^+ currents had the characteristics of a slow time course, and interestingly, α-PMTX was found to have no effect on the TTX-resistant Na^+ channels in TG neurons (Fig. 6B). We tested the effects of α-PMTX on cultured hippocampal pyramidal neurons and found that the toxin also slowed Na^+ currents (Fig. 6C). The I_{ss}/I_{peak} percentage ratios were 1.7 ± 0.5 (mean ± SEM; $n = 14$) in the control and 6.6 ± 2.1 ($n = 10$) with α-PMTX in the hippocampal neurons. The effects of ATXII on Na^+ currents in the TG neuron and hippocampal neurons appeared to be similar to those of α-PMTX; the I_{ss}/I_{peak} percentage ratios were 1.0 ± 0.3 and 13.6 ± 1.9 ($n = 4$) before and after application of ATXII (1 μM) to TG neurons, respectively. In the hippocampal neurons, the values were 3.3 ± 0.8 ($n = 12$) in the control and 11.1 ± 1.9 ($n = 6$) with ATXII. ATXII slowed TTX-sensitive Na^+ currents but had no effect on TTX-resistant Na^+ currents in TG neurons (Fig. 6D and F).

Chemical modification of α-PMTX

We prepared a number of synthetic analogues of α-PMTX and investigated its structure–activity relationship on the lobster neuromuscular synapse (Fig. 7). When the NH_2-terminus of α-PMTX was replaced with an OH-terminus, the activity was completely lost. Deletion of the first five amino acids (PMTX 6–13) or the last four amino acids (PMTX 1–9) also resulted in loss of the activity. Replacement of isoleucine at position 2 with phenylalanine (F2I), or phenylalanine at position 7 with tyrosine (Y7F) abolished the activity. In contrast, when arginine at position 12 was changed to lysine, the potency increased. The effective concentration of inducing repetitive action potentials was ~five times lower than that of α-PMTX. The qualitative nature of repetitive action potentials caused by this mutant toxin was similar to α-PMTX. Interestingly, this toxin structure, which we named β-PMTX, is identical to a compound found in the venom of another species of the Pompilidae family (*Batozonellus maclifrons*, Konno *et al*., 1998).

Discussion

A wide range of neurotoxins have known modulatory actions on Na^+ channel function, including the processes responsible for channel activation and inactivation, ionic selectivity, and voltage-sensitive properties controlling gating and transition between functionally distinctive channel states. At least six groups of neurotoxin-binding sites on the Na^+ channel have so far been described (Catterall, 1980, 1995; Strichartz *et al*., 1987; Fainzilber *et al*., 1994). Regarding the effect of neurotoxins on the slowing of Na^+ channel inactivation, both α-scorpion toxins and sea anemone toxins decreased the steepness of voltage dependence of steady-state inactivation of Na^+ currents (West *et al*., 1992; Gallagher & Blumenthal, 1994; Khera *et al*., 1995), reduced the late components of the gating current and blocked the immobilization of gating charge that occurs following channel activation (Neumcke *et al*., 1985). More recently, Rogers *et al*. (1996) showed that a major determinant of LqTx and ATXII binding constituted Glu1613 and the extracellular S3–S4 loop at the extracellular end of the S4 voltage sensor in domain IV of the Na^+ channel α-subunit. Because the S4 segment in domain IV of the Na^+ channel was believed to move toward the extracellular space during depolarization, translocation of IV S4 was required for the inactivation gate to close (Catterall, 1995; Yang & Horn, 1995). Site 3 neurotoxins that were bound at the extracellular end of IVS4 could slow or block this translocation, preventing inactivation and gating charge immobilization (Rogers *et al*., 1996).

In the present study, we have demonstrated that α-PMTX derived from wasp venom acts on Na^+ channels by slowing the channel inactivation without changing the peak current–voltage relationship or the activation time course of the TTX-sensitive Na^+ currents. α-PMTX exhibited voltage-dependent actions with more rapid loss during stronger depolarization, similar to ATXII. Furthermore, the toxin showed voltage-dependent effects on the rate of recovery from inactivation and deactivating tail currents. All of these observations can be understood by assuming that α-PMTX slows the conformational changes that are required for fast inactivation, possibly by binding to common elements of the neurotoxin receptor site 3 on the extracellular surface of the Na^+ channel.

It is noteworthy that α-PMTX and ATXII acted quite differently on the lobster axon. Repetitive action potentials induced by α-PMTX in the lobster axon are not accompanied by the long-lasting membrane depolarization, which is invariably observed with ATXII or LqTx Iα. It is possible that ATXII or LqTx Iα may act not only on the Na^+ channels sensitive to α-PMTX but also on some K^+ channels, and their blockade may cause marked prolongation of the action potentials in the axon. However, this explanation is not very likely because ATXII was entirely ineffective on K^+ currents in dissociated neocortical neurons (Mantegazza *et al*., 1998) and LqTx Iα had no effect on K^+ conductance in insect axons (Eitan *et al*., 1990). It is more likely that the recovery from inactivation of the Na^+ channel is faster in the presence of α-PMTX at the lobster axon terminal. It has been reported that funnel web spider toxins, versutoxin and robustoxin, which have selective interaction with Na^+ channel gating and kinetics like α-scorpion toxins, increased the repriming kinetics of the Na^+ channels when the channels return to the resting state following activation (Nicholson *et al*., 1994, 1998). Similarly, in the present study, α-PMTX shortened the fast component of the rate of recovery from Na^+ channel inactivation at –80 mV (τ_f was 4.59 ms in the control and 1.28 ms in the presence of the toxin). Although the funnel web spider toxins produce a hyperpolarizing shift in steady-state inactivation, an action different from that of α-PMTX, the two types of toxins may share some part of their structures to exert similar actions. In line with this, it is assumed that toxins that bind to neurotoxin receptor site 3, e.g. sea anemone toxins, scorpion toxins, funnel web spider toxins and α-PMTX, may act on overlapping but not identical sites on the Na^+ channels. For example, multiple attachment sites of α-scorpion toxin binding have been identified, based on biochemical evidence, on the IS5–S6 and IVS5–S6 loops in the molecule (Tejedor & Catterall, 1988; Thomsen & Catterall,

1989). Therefore, the discrepancy in the effect of the neurotoxins in different preparations may be caused by the amount of expressed channel subunits that are specific for the toxin binding. It has become clear that Na^+ channels in various tissues differ in their functional properties and the diversity reflects the expression of distinct Na^+ channel genes in each tissue (Raman & Bean, 1997). Actually, the present study shows that α-PMTX slows Na^+ currents in cultured hippocampal neurons, but in the slice preparation, the toxin was more effective on the axonal membrane than the somal membrane of the CA1 pyramidal neurons (Yokota *et al*., 1998).

It is interesting to note that α-PMTX consists of only 13 amino acids (13 AA) and exhibits no structural homology with other toxins known to bind to neurotoxin receptor site 3, i.e. scorpion toxins (60–65 AA), sea anemone toxins (46–49 AA), robustoxin or versutoxin (42 AA) and δ-conotoxins (29–31 AA). Furthermore, α-PMTX lacks disulphide bonds which are present in abundance in other toxins. Sea anemone toxins or scorpion toxins contain several disulphide bonds which render their structure rigid (Fontecilla-Campus *et al*., 1980), and a reduction in number of these disulphide bonds appears to destroy their biological activities (Loret *et al*., 1994). It is possible that α-PMTX is part of a high-molecular polypeptide in the venom. However, in the process of purification of the venom extract using high-performance liquid chromatography (HPLC), we tested the biological activity of each fraction on lobster neuromuscular preparation and failed to find similar activity as α-PMTX in high-molecular fractions (Konno *et al*., 1998). Therefore, α-PMTX may not be a degradation product of a larger peptide in the venom.

We have shown that replacement of a certain amino acid in the sequence of α-PMTX caused dramatic changes in its toxic activity. More complete molecular data on the toxin, including the global charge, examination of electrostatic surface potential and modelling structure of α-PMTX are necessary. The simple structure of α-PMTX would offer a special advantage for further mutation studies to elucidate the molecular mechanism by which Na^+ currents are modified, and to characterize different types of Na^+ channels.

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Abbreviations

4AP, 4-aminopyridine; EDTA, ethylene diamine tetraacetic acid; EPSP, excitatory postsynaptic potential; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulphonic acid]; HPLC, high-performance liquid chromatography; IPSP, inhibitory postsynaptic potential; α-PMTX, α-pompilidotoxin; TG, trigeminal ganglion; TTX, tetrodotoxin.

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