Characterization of the palytoxin-induced sodium conductance in frog skeletal muscle

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- 1 The effects of palytoxin (PTX) on transmembrane potentials and currents of frog skeletal muscle were analyzed by intracellular microelectrode techniques and the double sucrose-gap voltage clamp method.
- 2 PTX irreversibly depolarized the membrane. The depolarization was Na-sensitive.
- 3 Under voltage clamp, PTX induced an inward resting current which did not inactivate, was inhibited by external Na⁺ removal and was a function of external Na concentration.
- 4 This resting current could be carried either by Na⁺, Li⁺, K⁺ or by guanidinium according to the permeability sequence K⁺ < Li⁺ < Na⁺ < Gua⁺.
- 5 The PTX-induced current was only weakly sensitive to tetrodotoxin. It was reversibly and dose-dependently inhibited by amiloride with a one to one stoichiometry and a K_D of 0.3 mM.
- 6 Acidic pH partially inhibited the current induced by PTX which was also highly sensitive to external Cd²⁺ and La³⁺. The inhibitory sequence for divalent cations was: Mg²⁺ < Ca²⁺ = Ba²⁺ = Mn²⁺ < Cd²⁺; with La³⁺ > Cd²⁺.
- 7 The amplitude of the PTX-induced I_{rest} was markedly reduced in the absence of external Ca²⁺.
- 8 PTX induced a Na⁺ resting conductance in frog skeletal muscle. The size of the channel induced by PTX is larger than the guanidinium ion. External membrane Ca²⁺ might be a cofactor involved in the mode of action of PTX.

Introduction

Palytoxin (PTX), a toxin isolated from a marine coelenterate by Moore & Scheuer, (1971), causes an irreversible sodium-dependent depolarization in many excitable cells (Dubois & Cohen, 1977; Sauviat & Pichon, 1977; Weidmann, 1977; Pichon, 1982; Tesseraux *et al.*, 1983; Sauviat *et al.*, 1987). It has been proposed that PTX acts as a Na ionophore in both *Torpedo* membrane vesicles (Stengelin *et al.*, 1983) and cardiac muscle (Sauviat *et al.*, 1987; Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988). In the cockroach giant axon the PTX-induced depolarization was decreased in the presence of tetrodotoxin (Sauviat & Pichon, 1977; Pichon, 1982) while in frog nerve, it was sensitive to saxitoxin (Castle & Strichartz, 1988). Although it appears that sodium plays a central role in the action of PTX, the mechanism by which the toxin depolarizes excitable cells remains unidentified. The goal of the present work was to characterize the toxin-induced conductance in frog skeletal muscle membrane and to identify its mode of action.

Methods

Voltage clamp experiments were performed at 10–12°C on fine, cut end skeletal muscle fibres (100–200 µm in diameter, 5–6 mm in length) isolated from sartorius muscle of *Rana esculenta*. The double sucrose-gap technique with vaseline seals was used (Pater & Sauviat, 1987). The membrane voltage was held at –80 mV and the resting current (I_{rest}) recorded. In experiments performed on intact cleaned sartorius muscle, conventional intracellular microelectrode techniques were used (electrode resistance 20 to 30 MΩ; tip potential ≤ ±3 mV) to record transmembrane potentials with a differential voltage follower and a fast flow experimental chamber similar to the recording system described by Sauviat (1990). The permeability ratio between sodium (Na⁺) and the substi-

tuted ion (X⁺) was calculated according to the Goldman-Hodgkin-Katz equation:

$$\Delta E_{rev} = RT/zF \ln(P_{X^+}[X^+]_o)/(P_{Na^+}[Na^+]_o)$$

in which ΔE_{rev} was the reversal potential difference between the two ions; P_{X⁺}/P_{Na⁺}, the permeability ratio; [X⁺]_o and [Na⁺]_o, the external concentration of both ions; T the absolute temperature; F the Faraday constant; z the valency of the ion and R the perfect gas constant.

It should be noted that each figure is representative of five to six experiments unless otherwise stated. Results are expressed as mean values ± s.e. of the mean; (n) corresponds to the number of preparations tested.

Transmembrane potentials and currents were displayed on an oscilloscope (Nicolet 310) and recorded on the mass storage unit of a desk-top computer (Hewlett Packard 9826) and plotted on an XY plotter (Ifelec or Hewlett Packard 7470) for further analysis.

The composition of the Ringer solution was (mM): NaCl 110.5, KCl 2.5 and CaCl₂ 2. The pH of the solution was maintained at 7.2 with HEPES buffer (5 mM). In experiments where differences in pH were studied, Tris acetylglycine KOH buffer (5 mM) was used to provide acidic pH and more basic pH values of 8 and 9.6 were obtained by use of TES buffer (5 mM) and NaHCO₃/NaOH buffer (5 mM) respectively. In Na-free solution, NaCl was isosmotically replaced by mannitol or, in some experiments by guanidinium chloride, LiCl or KCl. Tetrodotoxin (TTX) was used in the range 10^{–7} to 10^{–5} M. Cadmium, barium, magnesium, manganese and lanthanum were used as CdCl₂, BaCl₂, MgCl₂, MnCl₂ and LaCl₃ respectively. Calcium-depleted solutions were made by simply omitting CaCl₂ from the composition of the Ringer solution and in some experiments by addition of 10^{–4} M ethylene glycol tetraacetic acid (EGTA) to the calcium-depleted solution. The composition of the internal perfusing solution was (mM): K aspartate 120, ATP 5, phosphocreatine 5, MgCl₂ 2, HEPES buffer 5, pH 7.2 (Pater & Sauviat, 1987).

PTX was dissolved in distilled water at a concentration of 3.7 × 10^{–5} M and kept at 4°C. The stock solution was appropriately diluted just before use.

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Results

Characterization of the effect of palytoxin upon membrane resting potential and current and the effect of the external Na concentration

Figure 1a illustrates the effect of PTX on the membrane resting potential of an intact muscle. The toxin induced a slow and irreversible depolarization which reached a plateau. The resting membrane potential was decreased from -81.6 ± 1.7 mV in the control Ringer solution to -51.2 ± 5.2 mV in the presence of the toxin ($n = 8$). The depolarization induced by PTX was abolished when Na^+ ions were removed from the control solution.

Under steady-state voltage clamp conditions PTX (4×10^{-8} M) induced a marked inward resting current (I_{rest}) which did not inactivate and was abolished by the removal of Na^+ from the toxin-containing solution (Figure 1b). The addition of 25% of normal Na^+ to the sodium-free medium re-established an inward I_{rest} of smaller amplitude than control which was also suppressed upon return to sodium-free medium. Figure 1c shows that the inward I_{rest} induced by pre-washing the fibre with PTX was a function of $[\text{Na}^+]_o$. I_{rest} increased monotonically upon the addition of Na^+ and reached a plateau whatever the Na^+ concentration. In Figure 1d, the open circles represent a plot of I_{rest} induced by PTX against the logarithm of $[\text{Na}^+]_o$. It can be seen that I_{rest} shows no $[\text{Na}^+]_o$ -dependent saturation in the concentration-range studied. The representation of the logarithm of I_{rest} as a function of the logarithm of $[\text{Na}^+]_o$ (Figure 1d) indicated that experimental data are best fitted by a straight line with a slope of unity suggesting a first order relation between Na ions and the effect of PTX on the membrane. The voltage-dependence of the Na conductance induced by the toxin is illustrated in Figure 2b. Experimental data are best fitted by a straight line which intercepted the membrane potential axis at +48 mV value closed to the apparent reversal potential for Na^+ . The PTX-induced conductance determined from the slope of the I-V curves was $8.9 \pm 1.8 \mu\text{S}$ ($n = 9$).

Total replacement of Na^+ by guanidinium ions (Gua^+) in the Ringer solution containing PTX generated an inward I_{rest} the amplitude of which was larger ($154 \pm 50\%$, ($n = 4$) at 0 mV) than that recorded in the presence of Na^+ (Figure 2a). The current was abolished after removal of the permeating ion (Na^+ or Gua^+) from the perfusing solution. Figure 2b shows that experimental data obtained with Gua^+ are best fitted by a straight line which crossed the abscissae at +76 mV. With Li^+ instead of Na^+ in the control solution, the inward current induced by PTX was of smaller amplitude and the apparent reversal potential shifted by 9 mV towards less positive membrane potentials. Total replacement of Na^+ by K^+ in the Ringer solution also shows that K^+ are able to carry the inward resting current in the presence of PTX with an apparent reversal potential of 0 mV. Results are summarized in Table 1 and suggest the following permeability sequence $\text{K}^+ < \text{Li}^+ < \text{Na}^+ < \text{Gua}^+$. Table 1 also shows that the conductance induced by the toxin, smaller in the presence of Li^+ , was of similar magnitude when the current was carried by either Na^+ , K^+ or Gua^+ and suggested the following conductance sequence $\text{Li}^+ < \text{Gua}^+ < \text{K}^+ < \text{Na}^+$. In three experiments performed in a mannitol-containing Na^+ -free solution in the absence and in the presence of 2 mM Ca^{2+} , Ca^{2+} was found to carry an inward current in the presence of PTX with a conductance of $1.2 \pm 0.2 \mu\text{S}$ and an apparent reversal potential of $+38 \pm 4$ mV.

Effect of tetrodotoxin and amiloride on palytoxin induced conductance

Figure 3a shows that TTX (5.7×10^{-7} M) did not inhibit the depolarization of the resting membrane potential induced by PTX. Under voltage clamp conditions, 5.7×10^{-7} M TTX

Figure 1 Effect of palytoxin (PTX) on the resting membrane potential and resting current (I_{rest}). (a) PTX (2×10^{-7} M) in Ringer solution irreversibly depolarized the resting membrane potential recorded in a whole cleaned muscle with an intracellular microelectrode. The depolarization induced by the toxin was reversed when external Na was replaced by mannitol ($\text{Na} = 0$). Interruption of the trace: 3 min of continuous record have been omitted. (b) PTX (4×10^{-8} M) induced an inward resting current which was suppressed when Na^+ was removed from the Ringer solution. A subsequent application of Na^+ (25%) induced an inward current of smaller magnitude than in normal Ringer solution. ($\blacktriangle$) Na -free Ringer solution. The membrane potential was held at -80 mV 2 min before the toxin application. (c) Effect of variation of extracellular Na concentration ($[\text{Na}^+]_o$) on the inward resting current induced by PTX (4×10^{-8} M). I_{rest} induced by PTX was recorded during the application of: ($\circ$) 25%; ($\ast$) 50%; ($\bullet$) 75% of normal $[\text{Na}^+]_o$ from a Na -free solution ($\text{Na} = 0$). Points corresponded to a digitization of the original records every 2.5 s. (d) Upper curve ($\circ$): I_{rest} induced by PTX as a function of the external Na concentration (ordinates, right scale). Points and vertical bars represent the mean and s.e. of I_{rest} ($n = 6$). Lower curve ($\bullet$): logarithm of I_{rest} induced by PTX as a function of the logarithm of the external Na concentration (ordinates, left scale). The holding potential was -80 mV in b, c and d.

Figure 2 Selectivity of inward resting current induced by palytoxin (PTX, 4×10^{-8} M). (a) Effect of the addition of 110 mM Na (upper traces) or 110 mM guanidinium (Gua; lower traces) to the Na-free Ringer solution ($\text{Na} = 0$; $\blacktriangle$) containing the toxin, applied 5 min before each run, on the inward resting current induced by PTX recorded at 0 mV holding potential. (b) Current-voltage relationships for the inward current recorded as in (a). ($\circ$) Ringer solution; ($\bullet$) solution containing guanidine.

Figure 3 Effect of tetrodotoxin (TTX) on the palytoxin (PTX)-induced resting potential and inward resting current (I_{rest}) recorded in the normal Ringer solution. (a) TTX (6×10^{-7} M) did not suppress the depolarization induced by PTX (2×10^{-7} M). (b) Effect of successive (i, ii, iii) application of TTX (10^{-5} M; $\bullet$) on the PTX (4×10^{-8} M)-induced inward I_{rest} recorded at -80 mV holding potential. PTX was first applied 10 min before starting the experiment.

slowed the development of the inward I_{rest} induced by PTX (not shown). At a higher concentration, TTX (1×10^{-5} M) induced a small and reversible block of the inward I_{rest} (Figure 3b).

The addition of amiloride to the Ringer solution containing PTX rapidly and dose-dependently inhibited the inward I_{rest} induced by the toxin (Figure 4). After amiloride washout, the current recovered.

Effect of external pH on palytoxin-induced conductance

Fibres were first perfused with a sodium-free Ringer solution containing PTX and then solution containing Na was applied for each pH studied. Figure 5a shows that an inward I_{rest} developed monotonically and reached a plateau whatever the pH tested in the presence of Na^+ . After sodium removal, the current rapidly recovered its initial value. Lowering the pH in the range 7.2 to 6.3 decreased the amplitude of the inward I_{rest} while increasing the pH in the range 7.2 to 8 increased it. The dose-response curve plotted for effect of external pH upon I_{rest} indicated that the amplitude of the current, taken as 100% at pH 7.2, is reduced by 42% at pH 5.5 and increased by 40% at pH 9.8 (Figure 5b). Figure 5b also shows that I_{rest} recorded in the absence of toxin depends weakly upon the external pH.

Table 1 Selectivity of the palytoxin (PTX, 4×10^{-8} M)-induced inward resting current in frog skeletal muscle fibres

Ion		Conductance (μS)	E_{rev} (mV)	Voltage-dependent Na channels			Ionic radius ($\text{\AA}$)
				$P_{\text{Li}}/P_{\text{Na}}$	$P_{\text{K}}/P_{\text{Na}}$	$P_{\text{Gua}}/P_{\text{Na}}$	
Na	(n = 9)	8.9 ± 1.8	$+50.0 \pm 6$	1.00	1.00	1.00	0.95
Li	(n = 6)	5.0 ± 1.2	$+41.0 \pm 3$	0.69	0.96	0.87	0.60
K	(n = 5)	7.9 ± 1.0	$+1.6 \pm 1$	0.019	0.086	1.11	1.33
Gua	(n = 5)	7.0 ± 3.0	$+68.0 \pm 6$	2.09	0.093	1.59	2.40
					(1) (frog muscle)	(2) (end plate)	

The resting inward current induced by PTX was measured at different holding potentials, the slope conductance and the apparent reversal potential were determined as in Figure 2. The permeability ratio ($P_{\text{Li}}/P_{\text{Na}}$) was calculated according to the Goldman-Hodgkin-Katz equation (see Methods).

The permeability ratio (1) and (2) are taken from Hille (1984). The ionic radius are given by Edwards (1982) and Hille (1984).

Figure 4 Effect of amiloride on the inward resting current induced by palytoxin (PTX, 4×10^{-8} M) at -80 mV holding potential. (a) Effect of successive applications of amiloride (0.25 mM; 0.5 mM; 0.75 mM). (b) Dose-response curve for the inhibition of the PTX-induced I_{rest} by amiloride. The line fitted to the data was estimated from the Hill equation with K_D of 0.34 mM and a slope of 1.02. Vertical lines represent s.e.mean of $n = 6$.

The logarithmic representation of I_{rest} as a function of the external pH was best fitted by a straight line with a slope of 0.03 in the absence and 0.45 in the presence of PTX.

Effect of external cations on palytoxin-induced conductance

Figure 6a shows that the addition of 20 mM Mn^{2+} to the Ringer solution containing PTX induced a moderate ($11.8 \pm 6.1\%$; $n = 4$) transient and reversible reduction of the inward I_{rest} . Increasing the external Ca^{2+} , Ba^{2+} or Mg^{2+} concentrations to 20 mM in the solution containing the toxin only transiently reduced the inward I_{rest} by $14 \pm 8\%$ ($n = 4$), 16.6 ± 1.4 ($n = 3$) and $3.6 \pm 1\%$ ($n = 3$) respectively. The addition of 6 mM Cd^{2+} to the Ringer solution containing PTX markedly and durably inhibited I_{rest} (Figure 6a). The inhibitory effect of Cd^{2+} was dose-dependent (Figure 6b) and saturated at concentrations larger than 10 mM. The inhibition reached 50% at about 3 mM Cd^{2+} . Figure 6c shows that the addition of 2 mM La^{3+} to the solution containing PTX also inhibited ($52.5 \pm 1.4\%$; $n = 4$) the inward I_{rest} which recovered rapidly after La^{3+} removal.

Figure 6d shows that a pretreatment of the fibre with Cd^{2+} did not prevent the effect of PTX though the inward I_{rest} was of smaller amplitude than that induced by the toxin in the absence of Cd^{2+} and the rate of development of the current in the presence of Cd^{2+} (1.0 ± 0.3 nA s $^{-1}$; $n = 3$) was decreased compared with the value obtained in the absence of Cd^{2+} (4.4 ± 0.2 nA s $^{-1}$; $n = 10$). Figure 6d also shows that removal of Cd^{2+} from the solution containing PTX led to an immediate increase of the amplitude of I_{rest} which was inhibited again when Cd^{2+} was reapplied.

Figure 5 Effect of external pH on the inward current induced by palytoxin (PTX, 4×10^{-8} M) recorded at -80 mV holding potential. (a) The resting current induced by PTX was elicited by addition, for each pH tested, of 110 mM Na to the Na-free Ringer solution containing PTX at the chosen pH (Na = 0; $\blacktriangle$): (○) pH 6.3; (*) pH 7.2; (●) pH 8. The recovery phase of pH 7.2 has not been included for clarity since it lies between the other two curves. Points were digitized from the original records every 2.5 s. (b) The resting current magnitude recorded in the Ringer solution before (○) and after (●) PTX treatment as a function of the external pH. Vertical lines represent the s.e.mean of $n = 6$.

Effect of Ca depletion on the development of the PTX-induced resting conductance

Figure 7a shows that the inward I_{rest} induced by the toxin in the calcium-free solution was of much smaller amplitude than the current recorded in the presence of Ca^{2+} (see Figure 1b) and the rate of development was slowed to 0.29 ± 0.07 nA s $^{-1}$ ($n = 4$). In Figure 7b, the fibre was successively perfused with different solutions to reduce external membrane Ca^{2+} and to replace Ca^{2+} bound at the membrane surface by La^{3+} in order to study the effect of PTX on a calcium-depleted membrane (Figure 7b(i)). Figure 7b(ii) shows that the inward I_{rest} subsequently evoked by PTX was of very small amplitude compared with the current recorded in Ringer solution (Figure 1b) and the rate of the current development was even slower (0.16 ± 0.07 nA s $^{-1}$; $n = 3$) than that recorded in calcium-free solution.

Discussion

The present study shows that PTX depolarized the membrane in frog skeletal muscle by inducing an inward resting current (I_{rest}) which presented at least five properties. It was: (i) a func-

Figure 6 Effect of external cations on the inward resting current (I_{rest}) induced by palytoxin (PTX, 4×10^{-8} M). (a) Inhibitory effect of manganese (Mn; 20 mM) and cadmium (Cd; 6 mM) on I_{rest} induced by 8 min of PTX application. (b) Dose-response relation for the inhibitory effect of cadmium (Cd) on the inward I_{rest} induced by PTX. The line was fitted by eye. (c) Reversible blockade of the inward I_{rest} recorded by the addition of 110 mM Na in the Na-free Ringer solution containing PTX (Na = 0; $\blacktriangle$) by lanthanum (La; 2 mM). After recovery I_{rest} was suppressed by Na⁺ removal from the Ringer solution. (d) Effect of PTX on the inward current in the presence of Cd²⁺ (20 mM; $\bullet$). (i) The membrane was bathed in a Ringer solution containing Cd²⁺ (20 mM). (ii) PTX was added to the control solution. (iii) Cd²⁺ was removed from the solution containing PTX, added again in (iv) and removed in (v). Holding potential was -80 mV in a, b, c, d.

Figure 7 Effect of palytoxin (PTX, 4×10^{-8} M) on the resting current recorded at -80 mV holding potential after external Ca depletion. (a) Effect of PTX in Ca-free solution. The fibre had been bathed for 5 min in a Ca-free Ringer solution before PTX was added. (b) Effect of PTX on a Ca²⁺-free and La³⁺-treated membrane. (i) The membrane was bathed for 5 min each in a Ca²⁺-free Ringer solution, Ca²⁺-free solution containing 10^{-4} M ethylene glycol tetraacetic acid (EGTA), Ca²⁺-free solution, Ca²⁺-free solution containing La³⁺ (2 mM), Ca²⁺-free solution and finally Ca²⁺-free solution containing PTX. (ii) Continuation of trace (i) showing the development of the PTX current in Ca²⁺-free solution and its suppression by removal of Na⁺ from the solution containing the toxin. Dashed lines represent the zero current in (a) and (b).

tion of [Na⁺]_o; (ii) highly permeable to guanidine and partially blocked by tetrodotoxin and amiloride; (iii) sensitive to external pH; (iv) reversibly inhibited by the cations Cd²⁺ and La³⁺; (v) markedly reduced by external calcium depletion.

The inward I_{rest} induced by PTX was a direct function of [Na⁺]_o and suggested that PTX either forms a channel by which Na⁺ enters the cells as suggested in frog heart muscle (Sauviat *et al.*, 1987) and mammalian cardiac myocytes (Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988) or activates an existing, inactive Na⁺ channel.

Li⁺, considered to be a fairly good substitute for Na⁺ as far as the TTX-sensitive Na channel is concerned ($P_{Li^+}/P_{Na^+} = 0.96$ in frog muscle according to Campbell, 1976), was less permeant (permeability ratio of 0.69) than Na⁺ through the PTX-induced channel (see Table 1). A similar result has been reported in squid giant axon ($P_{Li^+}/P_{Na^+} = 0.62$) according to Muramatsu *et al.* (1984) and the frog heart (Sauviat *et al.*, 1987). Guanidinium ions which are considered as only weakly permeant through the voltage-dependent Na channel (Hille, 1975; Benoit & Dubois, 1987), are able to generate an inward current in the PTX-treated fibres. The permeability ratio P_{Gua^+}/P_{Na^+} of 2.09 was larger than the ratio reported for the voltage-dependent Na channel of the skeletal muscle of the frog (0.093; Campbell, 1976) or mammal (0.16; Pappone, 1980). It should be noted that a high permeability ratio for guanidine (1.59) has already been reported for the endplate Na⁺ channel of the frog (Dwyer *et al.*, 1980). However, the endplate sodium channel allows Na⁺ and K⁺ to cross the membrane with nearly equal ease (Adams *et al.*, 1980) while, in the PTX-induced one, K⁺ are only weakly permeant (Table 1). This suggests that the PTX-induced conductance did not use the known voltage-dependent Na channel but that PTX

formed its own channel. From the present results, the following selectivity sequence is proposed for the PTX-induced conductance: $K^+ < Li^+ < Na^+ < Gua^+$. This allows us to suggest that the size of the PTX-induced pore should thus be at least as large as the diameter of Gua^+ , estimated to be 4.8 Å (Edwards, 1982). Such a pore size might account for the permeability of large organic cations (hydroxymethylamine, methylhydroxylamine, methylamine, ammonium) and Cs^+ reported by Castle & Strichartz, (1988) in frog sciatic nerve and Ca^{2+} in the present work. However, pore size does not directly determine the conductance which is mainly determined by binding of ions to the channel. The conductance sequence ($Li^+ < Gua^+ < K^+ < Na^+$) is different from the permeability sequence ($K^+ < Li^+ < Na^+ < Gua^+$) indicating that permeating ions bind to the channel. This observation strongly suggested the existence of binding sites in the channel which attract ions when they pass from one side of the membrane to the other.

Due to the existence of a guanidinium group in tetrodotoxin and amiloride, it is tempting to think that this is responsible for the effect of these molecules on the PTX-induced conductance. Tetrodotoxin, at about hundred times the concentration required to inhibit entirely voltage-dependent Na channels in frog muscle (Pater & Sauviat, 1987), only reduced the PTX-induced current. The rapid onset of inhibition and the rapid reversal suggested that amiloride blocked the inward I_{rest} induced by PTX by acting from the outside of the membrane. The unity stoichiometry of the reaction amiloride- I_{rest} indicated a relation between one amiloride molecule and one PTX pore. Amiloride perhaps plugs the PTX formed channel by means of its guanidine group which interacts with the Na^+ site, as is generally proposed for the blocking action of the molecule on the Na channel in epithelial membranes (Sariban-Sohraby & Benos, 1986; Eaton & Hamilton, 1988). We should emphasise that the selectivity of the amiloride-blockable Na^+ channel is different from that induced by PTX in the sense that Li^+ could traverse the channel more easily than Na^+ while ammonium, hydroxylammonium, hydrazinium and Cs^+ are essentially impermeant (Garty & Benos, 1988). The rapid inhibition and recovery of the PTX-induced current by TTX and amiloride suggested that both molecules do not bind to a specific site or receptor at the PTX channel input but behave mainly as simple cationic molecules. The results do point out the existence of an antagonism between PTX and TTX in frog muscle as well as in the giant axon of the cockroach (Sauviat & Pichon, 1977; Pichon, 1982). Moreover, Castle & Strichartz, (1988) have shown that saxitoxin, an analogue of TTX, reduced the depolarizing effect of PTX in frog nerve.

Acidic pH solution is known to inhibit the voltage-dependent Na conductance in heart (Chesnais *et al.*, 1975) and skeletal muscle (Campbell & Hille, 1976). Although no apparent effect of pH in the range 6.2 to 8.4 on the PTX-induced depolarization was found in frog nerve (Castle & Strichartz, 1988). We observed that acidic pH solution blocked the PTX-induced current whereas, basic pH facilitated the conducting property of the PTX pathway in skeletal muscle. According to Frelin *et al.* (1990), PTX increased or decreased the intracellular pH depending on the value of extracellular pH in chick embryonic ventricular cells suggesting that PTX opened a pre-existing H^+ conductive pathway. In the present study, PTX modifies by a factor of about ten the slope of the relation between the resting current and the external pH indicating that the PTX-induced conductance decreased with the proton activity. This might suggest that the toxin activates the Na^+/H^+ antiporter whose sodium flux is known to be amiloride-sensitive. Although the antiporter can be activated by the toxin via a mechanism that is distinct from that of phorbol ester (Frelin *et al.*, 1990), it cannot be considered as the only process responsible for the Na influx induced by PTX since the ionic selectivity of the PTX-induced conductance differed from the selectivity of the Na^+/H^+ exchanger; Li^+ is a good substitute for Na^+ and Cs^+ presents no appreciable affin-

ity for the exchanger (Mahnensmith & Aronson, 1985), whereas, concerning the PTX-induced conductance, Li^+ is a relatively poor substitute for Na (this work) and Cs^+ is almost as permeable as Na^+ (Castle & Strichartz, 1988). On the other hand, the perfect reversibility of the effect of external pH changes also suggest that pH might (i) induce changes in the molecular configuration around a pKa value, (ii) alter the insertion of the molecule into the membrane, (iii) modify the degree of availability of negative surface charges and (iv) alter the amount of Ca^{2+} bound to the external side of the membrane. Low pH is known to displace Ca^{2+} from the phospholipids phosphate and carboxyl groups (Seimiya & Ohki, 1973; Hille *et al.*, 1975).

Divalent cations inhibited the PTX-induced Na current. The following sequence for the inhibitory effect of the divalent cations can be deduced: $Mg^{2+} < Ca^{2+} = Ba^{2+} = Mn^{2+} < Cd^{2+}$; La^{3+} was found to be more effective than Cd^{2+} . Cd^{2+} did not protect the membrane against PTX since the toxin-induced inward current developed immediately after Cd^{2+} removal, an observation suggesting that Cd effect occurred in the vicinity of the external surface of the membrane. According to Sorensen *et al.* (1985), Cd^{2+} might be able to occupy anionic sites on the phospholipid head group of the membrane which would otherwise be occupied by Ca^{2+} . La^{3+} is known to replace Ca^{2+} and to bind firmly and almost irreversibly to the membrane (Langer & Franck, 1972). Experiments in which the membrane has previously been depleted of Ca^{2+} and bathed in a solution containing La^{3+} , show that the effect of PTX is markedly delayed and smaller than in the presence of Ca^{2+} and suggested that PTX binds to the membrane even when it has been depleted of Ca^{2+} and when negative surface charges have been occupied by Cd^{2+} or La^{3+} . This suggests that external membrane Ca^{2+} plays an important role in the action of PTX and might appear as a cofactor. It could be suggested that Ca^{2+} either activated an unidentified subunit of the toxin or facilitated the formation of a dimer, since two molecules of PTX are involved in the resting Na^+ conductance development of the frog heart muscle (Sauviat *et al.*, 1987) and in neuroblastoma cells (Rouzaire-Dubois & Dubois, 1990). On the basis of the present results, the following model might be proposed to account for the mode of action of PTX:

PTX first binds either to the membrane phospholipids or an unidentified membrane receptor called (m) and forms a complex (PTXm) which is responsible for the poor conducting state observed in calcium-depleted membranes (Figure 7). Then two nearby PTXm are associated or activated by external Ca^{2+} which behave as a cofactor and lead to the development of the high conducting state (2PTXm). This high conducting state can be deactivated by external Cd^{2+} or La^{3+} , and drive back to the low conduction state (PTXm).

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