3,4 Dichlorobenzamil-sensitive, monovalent cation channel induced by palytoxin in cultured aortic myocytes

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1 Smooth muscle cells were dispersed from rat aorta and then cultured. The action of palytoxin on rat aortic myocytes was analysed by measurement of $^{22}\text{Na}^+$ uptake and single channel recording techniques.

2 Palytoxin induced an increase in $^{22}\text{Na}^+$ uptake, with a concentration of 50 nM producing half-maximal activation. The action of palytoxin was inhibited by amiloride derivatives and by ouabain. The concentrations of inhibitor producing half-maximal inhibition were 10 μM for 3,4 dichlorobenzamil, 30 μM for benzamil, 100 μM for phenamil and 1 mM for ouabain.

3 In outside-out patches, palytoxin induced single channel currents that reversed near 0 mV with NaCl or KCl in the extracellular solution, but were outward with N-methyl-D-glucamine chloride or CaCl_2 (110 mM), indicating that palytoxin induced a cation channel permeable to Na^+ and K^+ ($P_{\text{K}}/P_{\text{Na}} = 1.2$) but not to Ca^{2+} ($P_{\text{K}}/P_{\text{Ca}} > 30$) or to N-methyl-D-glucamine (NMDG) ($P_{\text{K}}/P_{\text{NMDG}} > 11$). The unit channel conductance was 11–14 pS.

4 A high (> 0.1 mM) extracellular concentration of Ca^{2+} was necessary to observe channel activation by palytoxin. A high (150 mM) extracellular concentration of K^+ partially prevented and reversed channel activation by palytoxin.

5 The channel activity was fully blocked by 3,4 dichlorobenzamil (20 μM) and partially blocked by phenamil (50 μM). It was not reduced by ouabain (200 μM).

Keywords: Palytoxin; vascular smooth muscle cell; non-selective cation channel; Na^+ influx; 3,4 dichlorobenzamil

Introduction

Palytoxin (PTX) consists of a long aliphatic, partially unsaturated chain with interspersed cyclic ether, hydroxyl and carboxyl groups (Moore *et al.*, 1982). It is one of the most potent toxins known and acts by impairing the function of excitable cells such as neuronal cells (Dubois & Cohen, 1977; Pichon, 1982) and cardiac cells (Sauviat *et al.*, 1987). PTX induces profound vasoconstriction and increases systemic blood pressure (Ito *et al.*, 1977). In different vascular preparations, PTX induces long lasting contractions that are partially sensitive to L-type Ca^{2+} channel antagonists, to indomethacin and to prazosin, an antagonist of α_1 -adrenoceptors (Karaki *et al.*, 1985). Smaller contractions are observed in the presence of an intact vascular endothelium and these can be increased by methylene blue (Nagase *et al.*, 1987). Taken together these results suggest that the vascular action of PTX involves (i) a direct action on vascular smooth muscle cells, (ii) the release of noradrenaline by nerve terminals (Karaki *et al.*, 1985; Nagase & Karaki, 1987), and (iii) the release of vasoconstrictor prostaglandins (Nagase & Karaki, 1987). These vasoconstrictor responses are partly counteracted by nitric oxide released by vascular endothelial cells (Nagase *et al.*, 1987).

Although it is clear that Na^+ plays a central role in the action of PTX (Habermann, 1989; Sauviat, 1992) the exact mechanism by which PTX acts remains to be established. Habermann proposed that PTX binds to the (Na^+ , K^+) ATPase and converts the enzyme into an open channel that allows Na^+ influx and K^+ efflux (Habermann, 1989). In excitable cells, the depolarization consecutive to the increased membrane permeability to Na^+ may trigger Ca^{2+} uptake by L-type voltage-dependent Ca^{2+} channels (Habermann, 1989). In cardiomyocytes (Ikeda *et al.*, 1988; Muramatsu *et al.*, 1988), in frog erythrocytes (Kim *et al.*, 1991) and in neuro-

blastoma cells (Rouzair-Dubois & Dubois, 1990), PTX opens a non-selective cation channel that is permeable to Na^+ and K^+ . Recent attention has been given to the action of various amiloride derivatives on PTX actions. In chick cardiomyocytes, PTX increases the permeability to H^+ , thereby leading to an intracellular acidification, and to a secondary activation of the Na^+/H^+ antiporter (Frelin *et al.*, 1990b). As a consequence, part of the PTX-activated $^{22}\text{Na}^+$ flux component could be inhibited by ethylisopropylamiloride (Frelin *et al.*, 1990b). The remaining PTX-activated $^{22}\text{Na}^+$ flux is blocked by 3,4 dichlorobenzamil, another derivative of amiloride that is well known to block the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity (Frelin *et al.*, 1990a). Finally, amiloride has been reported to block PTX-induced inward currents in frog skeletal muscles (Ecalt & Sauviat, 1991). In this study, we characterize PTX-induced channels in aortic myocytes with a combination of $^{22}\text{Na}^+$ uptake measurements and single channel current recordings, and define the properties of interaction of this channel with amiloride derivatives and with ouabain.

Methods

Cell culture

Wistar AF/Han rats (6–9 week old) were stunned and killed by cervical dislocation. The thoracic aorta was removed, cleared of fat and connective tissues and digested with collagenase and elastase as previously described (Van Renterghem & Lazdunski, 1991). Cells were grown in a M199 culture medium (GIBCO) supplemented with foetal bovine serum (GIBCO; 20%), glutamine (2 mM), penicillin (100 units ml^{-1}), and streptomycin (100 $\mu\text{g ml}^{-1}$) in collagen coated dishes (electrophysiological experiments) or regular culture dishes ($^{22}\text{Na}^+$ uptake experiments). For $^{22}\text{Na}^+$ uptake experiments, stock cultures were trypsinized and passaged

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once a week and cells used at passages 2–10. No change in the responses to PTX was observed as cells were passaged *in vitro*. Sparse primary cultures (3–8 day old) or first passage cultures were used for electrophysiological experiments. Only dark cells with the spindle-shape morphology of contractile myocytes were chosen for patch recording.

Flux experiments

Cells, seeded into 24 well tissue culture clusters, were first equilibrated for 15 min at 37°C in a K^+ -free modified Earle's salt solution containing (in mM): NaCl 145, $CaCl_2$ 1.8, $MgCl_2$ 0.8, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)/tris-hydroxymethylaminomethane (Tris) 25. Cells were then switched to a K^+ -free modified Earle's salt solution supplemented with $4 \mu Ci ml^{-1}$ $^{22}Na^+$ (Amersham; $0.5 Ci mg^{-1}$) and PTX. A K^+ -free solution was used to prevent $^{22}Na^+$ efflux by the $(Na^+, K^+)ATPase$. After 3 min of incubation at 37°C, the incubation solution was aspirated off and cells washed 4 times with $MgCl_2$ (0.1 M). Cells were then scraped off into NaOH (0.1 M) and the cell-associated radioactivity was counted. When the action of inhibitors was tested, inhibitors were added at the same time as PTX. The sensitivity of the $(Na^+, K^+)ATPase$ of aortic myocytes to ouabain was determined in $^{86}Rb^+$ uptake experiments. Cells in 24 well tissue culture clusters were loaded with Na^+ by equilibrating cells for 15 min in a K^+ -free Earle's salt solution supplemented with $10 \mu M$ monensin (Calbiochem). Cells were then placed for 3 min in an Earle's salt solution containing 5 mM KCl and supplemented with the desired concentrations of ouabain and $2 \mu Ci ml^{-1}$ $^{86}Rb^+$ (Amersham; $8 mCi mg^{-1}$). Cells were washed with $MgCl_2$ (0.1 M) and the cell-associated radioactivity was counted.

Protein contents were determined according to Bradford (1976).

Electrophysiological recordings and analysis

Single channel currents were measured (EPC 7, List Electronic, Darmstadt-Eberstadt, Germany) from excised patches in the outside-out configuration, using 12–17 M Ω patch electrodes. Data were recorded on a video tape recorder after digitization (44 kHz; PCM, Biologic, Grenoble, France), transferred to a microcomputer (Plessey systems, model 6220, Irvine, Calif., U.S.A.) after low pass filtering (see figure legends for bandwidth), and plotted with a digital plotter (Hewlett-Packard, Sunnyvale, Calif., U.S.A.). Single channel current amplitudes were measured from the gaussian fit of current density histograms. Linear fits were calculated by the least square regression method. The open state probability ($P(O)$) was measured from the gaussian fit of current density histograms. Inward currents (from extracellular face to intracellular face) are given negative values and represented downwards. All external solutions contained glucose (5 mM). The 'normal extracellular solution' at pH 7.4 contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES/NaOH 10. Reduced external Ca^{2+} concentration (0.1 mM; Figure 7) was obtained with a mixture of $MgCl_2$ (4.0 mM), $CaCl_2$ (1.30 mM), and ethylene glycol bis (β -aminoethyl ether), *N,N,N',N'* tetraacetic acid (EGTA) (1.20 mM). Free calcium concentration was computed as described previously (Fabiato & Fabiato, 1979). Other external solutions at pH 7.4 contained (in mM): $CaCl_2$ 2, $MgCl_2$ 1 plus KCl 150 and HEPES/KOH 10 ('150 mM K^+ solution'), or 150 N-methyl D-glucamine (NMDG) chloride and 10 HEPES/Tris ('150 mM NMDG solution'), or 108 $CaCl_2$ and 10 HEPES/ $Ca(OH)_2$ ('110 mM Ca^{2+} solution'). The 'pipette solution' at pH 7.2 contained (in mM): KCl 150, $MgCl_2$ 4, HEPES/KOH 10, EGTA 2, and $CaCl_2$ 1.79 or 0.16 (free Ca^{2+} ions concentration: 1 μM or 10 nM). Changing the internal Ca^{2+} concentration from 10 nM to 1 μM did not alter the properties of PTX-induced

channels. PTX was applied near the patch through a micro-perfusion system.

Means $\pm$ s.e.mean are indicated.

Toxin and drugs

3,4 Dichlorobenzamil (3,4 DCB) was synthesized as previously described (Vigne *et al.*, 1988). Ethylisopropylamiloride (EIPA), benzamil and phenamil were obtained from Dr E.J. Cragoe Jr. Palytoxin, from *Palythoa caribaeorum*, was kindly provided by Dr E. Habermann. It was dissolved in distilled water and kept frozen as 0.1 mM stock solutions. Final dilutions were made immediately before use.

Results

Palytoxin induces Na^+ uptake by aortic myocytes

PTX activated a $^{22}Na^+$ flux component in cultured aortic myocytes. The PTX concentration producing half-maximal activation was 50 nM (Figure 1a). Similar results were obtained with smooth muscle cells of the A7r5 cell line (not shown). The PTX-activated $^{22}Na^+$ influx component was prevented by 3,4 DCB, benzamil and by phenamil. The concentrations that produced half-maximal inhibition (IC_{50}) were 10 μM , 30 μM and 100 μM for 3,4 DCB, benzamil and phenamil respectively (Figure 1b). The action of PTX was reduced by less than 10% by amiloride (300 μM) or EIPA (300 μM). The PTX-activated $^{22}Na^+$ influx component was also inhibited by ouabain with an IC_{50} value at 1 mM (Figure 2). The dose-response curve for ouabain inhibition of the $^{86}Rb^+$ uptake by the $(Na^+, K^+)ATPase$ shows an IC_{50} value of 30 μM (Figure 2). These results indicate a 30 fold

Figure 1 Palytoxin (PTX) activates $^{22}Na^+$ uptake by aortic myocytes. (a) Dose-response curve for PTX action on $^{22}Na^+$ uptake by aortic myocytes. $^{22}Na^+$ uptake is expressed in d.p.m. $\times 10^{-5} min^{-1} mg^{-1}$ of protein. (b) Dose-response curves for 3,4 dichlorobenzamil (●) and phenamil (○) inhibition of the PTX (100 nM) activated $^{22}Na^+$ influx component in aortic myocytes.

difference in the actions of ouabain on the PTX-activated $^{22}\text{Na}^+$ influx and on the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ activity. The difference is probably larger than this value, since the action of ouabain is well known to be competitively antagonized by external K^+ . The sensitivity of the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ to ouabain was measured in the presence of external K^+ (5 mM). PTX-activated $^{22}\text{Na}^+$ influx was measured in K^+ -free solutions.

Palytoxin induces non selective cation channels in aortic myocytes

During patch recording from rat aortic smooth muscle cells, application of PTX to the extracellular membrane face commonly induced single channel inward currents (Figure 3). Inward currents following PTX (100 nM) application were observed in 34 out of 37 outside-out patches bathed in the 'normal extracellular solution'. Similar channels were observed following application of PTX to patches excised from aortic cells of the A7r5 cell-line (2 out of 3 patches, not shown). The number of channels activated was dependent on the concentration applied (4 patches), with a threshold concentration near 1 nM (Figure 3). This value is very near the threshold concentration for PTX-induced increase in $^{22}\text{Na}^+$ uptake by aortic myocytes (Figure 1a), suggesting that the channels observed in patch clamp experiments are probably responsible for the PTX-activated $^{22}\text{Na}^+$ flux component. PTX action was rapidly reversible upon washing (Figure 3c).

Figure 2 Dose-response curve for ouabain inhibition of the palytoxin (100 nM)-activated $^{22}\text{Na}^+$ influx component (O), and of $^{86}\text{Rb}^+$ uptake by the $(\text{Na}^+/\text{K}^+)\text{ATPase}$ (●).

Figure 3 Concentration-related induction by palytoxin (PTX) of single channel inward currents in outside-out patches. PTX 10 pM (a), 1 nM (b), or 100 nM (c,d) was applied for the time indicated by the bars (a–c). Holding potential, -30 mV. In (c), the maximal number of channels open in the patch was estimated from the single channel current amplitude to be 29. Opening of the first channels in (c) is shown on an expanded time scale (d). These recordings were obtained from a batch of cells presenting a particularly large response to PTX. Current scale bar: 2 pA, time scale bar: 1 min (a–c), or 5 s (d). Sampling: 10 Hz (a–c) or 500 Hz (d); filtering: 4 Hz (a–c) or 200 Hz (d). 'Normal external solution', and 'pipette solution' with 10 nM free Ca^{2+} .

The current/voltage relation for PTX-activated channels was linear (Figure 4). The single channel conductance was 10.8 ± 0.8 pS ($n = 9$), and the reversal potential was -2.7 ± 2.1 mV ($n = 9$). It was possible to observe PTX-induced single channel currents in patches exposed to symmetrical '150 mM K^+ solutions' (Figure 5a–b; see also Figure 8). The single channel conductance, measured from the current/voltage relations presented in Figure 5b was 14.3 ± 0.6 pS ($n = 4$). The reversal potential was -0.2 ± 0.8 mV ($n = 4$). Finally, when NMDG was substituted for inorganic monovalent cations in the extracellular solution, only outward currents were observed (Figure 5c–e). The current/voltage relation (Figure 5e) indicated a single channel conductance of 2.0 ± 0.1 pS ($n = 5$) and a reversal potential more negative than an extrapolated value of -60 mV.

Permeability ratios of the palytoxin-induced channel

Assuming that the contribution of divalent cations and chloride ions to the currents was negligible in the conditions of Figures 4 and 5, and using the Goldman, Hodgkin, Katz equation and the limit for the reversal potential (E_{rev}) value,

Figure 4 Current-voltage relation in the 'normal extracellular solution'. (a) Single channel currents recorded in the presence of palytoxin (PTX, 100 nM). Holding potentials: $+30$, $+10$, -10 , -40 , -60 , -80 , -100 , and -120 mV. Current scale bar: 0.8 pA (3 upper traces) or 2 pA, time scale bar: 1 s. Sampling: 1 kHz, filtering: 400 Hz. (b) Corresponding current-to-voltage relations obtained from individual outside-out patches. The single channel conductance value measured by linear fit of data between -120 and -10 mV (straight line) is 11.1 pS, and the reversal potential -3 mV.

Figure 5 Ionic selectivity of palytoxin (PTX)-induced channels. (a) Single channel currents recorded in the presence of PTX (100 nM) in the '150 mM K^+ solution'. Holding potentials: -30 , -50 , and -70 mV. (b) Corresponding current-voltage relations obtained from individual outside-out patches. The single channel conductance value measured by linear fit of data (straight line) is 14.2 pS, and the reversal potential 0 mV. (c,d) Single channel currents recorded in the presence of PTX (100 nM) in the '150 mM NMDG solution' (d), and current density histograms for currents recorded at -50 (left) and -20 mV (c). Holding potentials: 0 , -20 , and -50 mV. (d) Corresponding current-voltage relations obtained from individual outside-out patches. The single channel conductance value measured by linear fit of data between -30 and $+20$ mV (straight line) is 2.0 pS. Current scale bar: 1 pA (a) or 0.2 pA (d), time scale bar: 1 s. Sampling: 1 kHz, filtering: 400 Hz.

permeability ratios were estimated to $P_K/P_{Na} = 1.2$ and $P_K/P_{NMDG} > 11$. Assuming that the contribution of divalent cations to the currents was negligible, the limit for the reversal potential value ($E_{rev} < -60$ mV) obtained with the '150 NMDG solution' (Figure 5e) leads us to conclude the $P_K/P_{Cl} > 16$.

Palytoxin-induced channels are not permeable to Ca^{2+}

The permeability of PTX-induced channels to divalent cations was next examined. When 110 mM Ca^{2+} was substituted for monovalent cations in the extracellular solution, only outward currents were recorded through PTX-induced channels (Figure 6) even for holding potentials as negative as -60 mV. PTX (100 nM)-induced outward currents were detected in 4 out of 6 patches. The single channel conductance of outward currents with Ca^{2+} as the main extracellular cation was 1.6 ± 0.1 pS ($n = 4$), a value close to the value observed with NMDG (2.0 ± 0.1 pS; Figure 5e). Assuming that the contribution of chloride ions to the currents was negligible and using the limit value for E_{rev} ($E_{rev} < -70$ mV) obtained by linear extrapolation of the data obtained in the '110 Ca^{2+} solution' (Figure 6d), P_K/P_{Ca} was estimated to > 30 (from an extension of the Goldman, Hodgkin, Katz equation; Lakshminarayanaiah, 1984).

Extracellular Ca^{2+} is necessary for channel induction by palytoxin

It is known that PTX requires the presence of external Ca^{2+} for its activity (Habermann, 1989; Ecault & Sauviat, 1991; Rouzaire-Dubois & Dubois, 1990). Figure 7 shows that when

the Ca^{2+} concentration in the extracellular solution ($[Ca^{2+}]_o$) was reduced to 0.1 mM, application of PTX did not result in channel activity. Increasing $[Ca^{2+}]_o$ to 1 mM in the continued presence of PTX rapidly induced channel activity, and this effect could be reversed by returning $[Ca^{2+}]_o$ to 0.1 mM (Figure 7). Absence of channel induction by PTX (100 nM) with a low $[Ca^{2+}]_o$ (< 100 μ M) was observed in 19 patches. Reversible induction when switching from 100 μ M to 1 mM was observed in 3 patches.

Extracellular K^+ prevents and reverses channel induction by palytoxin

Figure 8a shows that when K^+ was substituted for Na^+ in the extracellular solution, cation channel induction by PTX occurred with only a very low efficiency. Such a very low efficiency of channel induction by PTX (100 nM) in the '150 mM K^+ solution' was observed in 11 out of 11 patches. Following channel induction in the normal extracellular solution, a sudden replacement of Na^+ by K^+ resulted in the closure of most channels within a few seconds (Figure 8b,c). This attenuation of the channel activity was observed in 6 out of 6 patches. Return to normal extracellular Na^+ was followed by a progressive reactivation of the channels within a few minutes (Figure 8b). The single channel amplitude was slightly increased following replacement of Na^+ by K^+ (see Figures 4b and 5d). Sudden replacement of Na^+ by Li^+ resulted in the closure of about two thirds of the channels within 3 min, and switching to Li^+ after channel closure by K^+ did not induce reactivation of the channels within 4–5 min (2 patches; not illustrated).

Figure 6 Non-permeability of palytoxin (PTX)-induced channels to Ca^{2+} ions. (a,b) Single channel currents recorded in the presence of PTX (100 nM) in the '110 mM Ca^{2+} solution'. Holding potentials: -50 (a), 0, -30, and -50 mV (b). Current scale bar: 0.2 pA, time scale bar: 1 min (a), 1 s (b). Sampling: 10 Hz (a), 1 kHz (b); filtering: 4 Hz (a), 400 Hz (b). (c) Current density histograms for currents recorded at -60 (left), -30 (middle), and +20 mV. (d) Corresponding current-to-voltage relations obtained from individual outside-out patches. The single channel conductance value measured by linear fit of data between -30 and +20 mV (straight line) is 1.6 pS.

Figure 7 Influence of extracellular Ca^{2+} on the channel activity. Currents recorded in the presence of palytoxin (PTX, 100 nM). 'Normal external solution', except that the concentration of Ca^{2+} was lowered to 0.1 mM during most of the experiment, as indicated. Holding potential: -30 mV. Current scale bar: 1 pA, time scale bar: 2 min. Sampling 7 Hz, filtering: 3 Hz.

Amiloride derivatives, but not ouabain, inhibit palytoxin-activated channels

Exposing outside-out patches to 3,4 DCB (20–50 μM) produced a period of flickering, followed by a quasi full block of PTX-activated channels (Figure 9a). The channel $P(O)$ was reduced by $95 \pm 5\%$ ($n = 6$). This observation is consistent with the fact that 3,4 DCB (20 μM) blocked more than 80% of the PTX-activated $^{22}\text{Na}^{+}$ uptake component (see Figure 1b). Phenamil (50 μM) also produced a flickering of the PTX-activated channel current (Figure 9b), but this was not followed by a closure of the channel. The channel $P(O)$ was reduced by $35 \pm 6\%$ ($n = 3$) in the presence of phenamil (50 μM). At this concentration, phenamil inhibited 16% of the PTX-activated $^{22}\text{Na}^{+}$ flux component (Figure 1b). Finally, ouabain (200 μM) had no effect on the PTX-activated

Figure 8 Influence of extracellular K^{+} on the channel activity. (a) Palytoxin (PTX, 100 nM) was applied for the time indicated in the '150 mM K^{+} solution'. (b) PTX (100 nM) was applied for the time indicated. The 'normal extracellular solution' was replaced by the '150 mM K^{+} solution' for the time indicated. Closure of the last channels following substitution of K^{+} for Na^{+} in experiment (b) is shown on an expanded time scale (c). Holding potential: -30 mV. Current scale bar: 2 pA, time scale bar: 1 min (a,b), or 1 s (c). Sampling: 10 Hz (a,b) or 50 Hz (c); filtering: 4 Hz (a,b) or 20 Hz (c).

currents within 5 min (Figure 9c), and the channel $P(O)$ was unchanged ($+2 \pm 2\%$, $n = 3$). At this concentration, ouabain had no action on the PTX-activated $^{22}\text{Na}^{+}$ flux component (Figure 2).

Discussion

This paper describes 3,4 DCB-sensitive, voltage-independent, Na^{+} and K^{+} permeable channels induced by PTX in aortic myocytes. The following observations indicate that PTX-induced channels were permeable to monovalent cations, with little ion selectivity. (i) Using the 'pipette solution' (KCl

Figure 9 Sensitivity to amiloride derivatives and not to ouabain of palytoxin (PTX)-induced channels. Single channel currents recorded in the presence of PTX (100 nM) in the 'normal extracellular solution'. Upper traces: control conditions. Lower traces: currents recorded starting 1 min (left) or 2 min after addition of 3,4 dichlorobenzamil (3,4 DCB, 20 µM) (a), or 3 min after addition of phenamil (50 µM) (b), or ouabain (200 µM) (c). Holding potential: -30 mV. Current scale bar: 0.5 pA, time scale bar: 1 s. Sampling: 1 kHz, filtering: 400 Hz. (d) Mean open state probabilities in the presence of 3,4 DCB, phenamil or ouabain expressed relative to their values before drug addition. Means $\pm$ s.e.mean are indicated.

150 mM), the corresponding currents reversed at -3 mV when recorded in the 'normal extracellular solution' (NaCl 150 mM, KCl 5 mM) (Figure 3b), and at 0 mV when recorded in the '150 mM K⁺ solution' (Figure 4b). (ii) When NMDG or Ca²⁺ were substituted for inorganic monovalent cations in the extracellular solution, only outward currents were recorded (Figure 4c-e and 5a-d). Taken together these results indicate that in rat aortic myocytes, PTX activates a cation channel that is permeable to Na⁺ and K⁺, but not to NMDG⁺ or to Ca²⁺. These properties are similar to those of the 9 pS channel previously shown to be induced by PTX in ventricular cardiomyocytes (Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988) and in frog erythrocytes (Kim *et al.*, 1991). The channels induced by PTX in both ventricular myocytes (Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988) and aortic myocytes (Figure 5a-d) have no measurable permeability to Ca²⁺. In mouse neuroblastoma cells, PTX also induces a non-selective cation channel, but its unit conductance is larger (26 pS) (Rouzaire-Dubois & Dubois, 1990). The permeability of PTX-induced channels in aortic myocytes to other monovalent cations was not investigated. In other cell types, PTX induces channels that are also permeable to NH₄⁺, Li⁺, Cs⁺ and guanidinium (Sauviat, 1992).

This paper also analyses the interaction of amiloride derivatives with PTX-induced channels. The PTX-activated ²²Na⁺ influx component was prevented by amiloride derivatives with the following rank order of potency: 3,4 DCB (IC₅₀ = 10 µM) > benzamil (IC₅₀ = 30 µM) > phenamil (IC₅₀ =

100 µM) >> amiloride, EIPA. Very similar results were obtained in electrophysiological experiments. 3,4 DCB, at a dose that inhibited 80% of the PTX-activated ²²Na⁺ flux component (Figure 1b) induced a flickering of the PTX-induced channels followed by their nearly complete blockade (Figure 9). Phenamil at a dose that inhibited 16% of the PTX-activated ²²Na⁺ flux component (Figure 1b) produced a flickering of the channel activity and a 35% reduction in the open state probability (Figure 9). 3,4 DCB was shown previously to prevent PTX-activated ²²Na⁺ uptake by chick cardiomyocytes with an IC₅₀ value (8 µM) similar to the one found in this study (10 µM) (Frelin *et al.*, 1990a). The pharmacological profile of action of amiloride derivatives on the PTX-induced channel differs from that on the epithelium Na⁺ channel for which phenamil is the more potent inhibitor (Barbry *et al.*, 1986). It is also distinct from that of the Na⁺/H⁺ antiporter for which EIPA is the most potent inhibitor (Vigne *et al.*, 1983).

3,4 DCB is the most potent inhibitor of PTX-induced channels found in this study. 3,4 DCB has been reported to inhibit a variety of membrane ion transport processes such as the Na⁺/Ca²⁺ exchanger (Siegl *et al.*, 1984; Kaczorowski *et al.*, 1985; Floreani *et al.*, 1987; Vigne *et al.*, 1988), L-type Ca²⁺ channels (Garcia *et al.*, 1990), and the (Na⁺,K⁺) ATPase (Floreani *et al.*, 1987). The IC₅₀ values for 3,4 DCB inhibition of the PTX-activated Na⁺ influx in aortic myocytes and in chick cardiomyocytes (8-10 µM) are similar to the IC₅₀ values reported for 3,4 DCB inhibition of the Na⁺/Ca²⁺ exchanger in neuronal cells (Kaczorowski *et al.*, 1985), in cardiac cells (Siegl *et al.*, 1984; Floreani *et al.*, 1987), and in aortic myocytes (Vigne *et al.*, 1988). Since PTX is known to induce a rise in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) in cardiomyocytes (Frelin *et al.*, 1990a), we previously proposed that the 3,4 DCB sensitive ²²Na⁺ uptake activated by PTX could result from an increased activity of the Na⁺/Ca²⁺ exchange system consecutive to a primary rise in [Ca²⁺]_i. The observation that 3,4 DCB inhibits PTX-induced channels in excised patches indicates however that the action of 3,4 DCB was unlikely to be a secondary action mediated by the Na⁺/Ca²⁺ exchanger. The ion-specificity of the PTX-induced channel differs also from that of the Na⁺/Ca²⁺ exchanger. First, Ca²⁺, one of the substrates of the Na⁺/Ca²⁺ exchanger hardly permeates through PTX-induced channels (Figure 6a-d). Second, K⁺, which is not transported by the Na⁺/Ca²⁺ exchanger, permeates through PTX-activated channels (Figure 5a,b). Although we cannot exclude the possibility that, in intact cells, a secondary activation of the Na⁺/Ca²⁺ exchange system may actually occur, our results suggest that 3,4 DCB has a direct action on PTX-induced channels.

Evidence has been presented that PTX may act through the (Na⁺,K⁺)ATPase in erythrocytes (Habermann, 1989). We show here that ouabain inhibited PTX-activated ²²Na⁺ uptake in aortic myocytes (Figure 2). The IC₅₀ value for ouabain inhibition (1 mM) is however more than 30 times as great as that for the inhibition of the (Na⁺,K⁺)ATPase activity as measured in ⁸⁶Rb⁺ flux experiments (30 µM) (Figure 2). We also observed in electrophysiological experiments that, at a concentration (200 µM) that inhibited 80% of the (Na⁺,K⁺)ATPase activity, ouabain had no action on the channel activity (Figure 9). These results suggest that the (Na⁺,K⁺)ATPase was unlikely to be the site of action of PTX in aortic myocytes.

A channel activity identical to that recorded in the presence of PTX has never been observed in aortic myocyte membranes in the absence of the toxin. This observation raises the hypothesis that PTX may act as an ionophore, by itself forming pores in the cell membrane. However PTX-induced channels share several properties with an epithelial-like Na⁺ channel recently described in aortic myocytes (Van Renterghem & Lazdunski, 1991). (i) The epithelial-like Na⁺ channel has a single channel conductance of 10 pS close to the value observed for PTX activated channels (11 pS, Figure

4). (ii) It is not voltage-dependent. (iii) It shows slow kinetics with very long openings (s). (iv) It is sensitive to high concentrations of amiloride derivatives (Van Renterghem & Lazdunski, 1991). These observations suggest the alternative hypothesis that PTX might act as an agonist of the epithelial-like Na^+ channel. Properties of the PTX-induced channel and the smooth muscle Na^+ channel differ however in some respects. First, whereas the epithelial-like channel is selective for Na^+ ($P_{\text{Na}}/P_{\text{K}} > 11$) (Van Renterghem & Lazdunski, 1991), the PTX-induced channel does not discriminate between Na^+ and K^+ ($P_{\text{Na}}/P_{\text{K}} = 0.9$). The difference in ion-selectivity does not mean necessarily that different structures are involved. It is known that activation of voltage-dependent, tetrodotoxin-sensitive Na^+ channels by a variety of neurotoxins is accompanied by a loss in the ion-selectivity (Khodorov & Revenko, 1979; Frelin *et al.*, 1981). Second, whereas epithelial-like Na^+

channels were most easily recorded in the cell-attached configuration, suggesting that as yet unidentified intracellular factors are required for their activity (Van Renterghem & Lazdunski, 1991), PTX-induced channels were currently recorded in excised patches with no evidence for a run down. It could be that PTX, like agonists of voltage-dependent channels, stabilizes an open conformation and bypasses the normal regulation of the channel activity.

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