Palytoxin Induces an Increase in the Cation Conductance of Red Cells

MAGDALENA T. TOSTESON, JOSE A. HALPERIN, YOSHITO KISHI, and DANIEL C. TOSTESON

From the Laboratory for Membrane Transport, Harvard Medical School, Boston, Massachusetts 02115; and Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT Palytoxin (PTX), isolated from the marine soft coral *Palythoa tuberculosa*, increases the cation conductance of human red cell membranes. In the presence of 10^{-10} M PTX and 10^{-5} M DIDS, the membrane potential approximates the equilibrium potential for Na^+ or K^+ rather than Cl^-. Even in the absence of DIDS, the Na^+ and K^+ conductances were greater than the Cl^- conductance. The selectivity of the PTX-induced cation conductance is $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+ \gg$ choline$^+$ > $TEA^+ \gg Mg^{2+}$. Measurements of K^+ efflux revealed two apparent sites for activation by PTX, one with a K_{a1} of 0.05 nM and a maximum flux, v_{max1}, of 1.4 mol/liter of cells per h and another with a K_{a2} of 98 nM and a v_{max2} of 24 mol/liter of cells per h. These effects of PTX are completely blocked by external ouabain (300 μM) and prevented by internal vanadate (100 μM). When the PTX channels are open, the Na,K pumps do not catalyze ATP hydrolysis. Upon thorough washout of cells exposed to about five molecules of PTX/pump, the Na,K pump of these cells operates normally. Blockage of the positively charged NH_2 terminus of PTX with a *p*-bromobenzoyl group reduces the potency of the compound to induce Na and K fluxes by at least a factor of 100, and to compete with the binding of [^{3}H]ouabain by at least a factor of 10. These data are consistent with the conclusion that PTX binds reversibly to the Na,K pumps in the red cell membrane and opens a (10-pS) channel equally permeable to Na and K at or near each pump site.

INTRODUCTION

Palytoxin (PTX) is an extremely potent toxin from marine coelenterates of the genus *Palythoa* (Moore and Scheuer, 1971). PTX has been shown to produce cation-selective channels in the plasma membranes of several different types of cells (Habermann, 1989, and references therein). The formation of these PTX-induced channels is inhibited competitively by ouabain and other cardiac glycosides (Ozaki, Nagase, and Urakawa, 1985). Moreover, PTX competes with ouabain for binding to purified

Address reprint requests to Dr. Magdalena T. Tosteson, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/91/11/0969/17 $2.00
Volume 98 November 1991 969–985

Na,K-ATPase and inhibits the enzyme (Ishida, Takagi, Takahashi, Satake, and Shibata, 1983; Grell, Lewitzi, and Uemura, 1988).

This article describes the results of experiments that characterize the action of the Okinawan PTX and of its terminal N-blocked derivative (NPB-PTX) on the cation permeability of human red cells. Confirming the observations of Habermann, Ahnert-Hilger, Chhatwal, and Béress (1981) on the actions of PTX extracted from *Palythoa caribaeorum*, we found that extremely low concentrations of PTX (1 pM) dramatically increase the permeability of the human red cell membrane to Na, K, and other monovalent cations. We report for the first time that exposure to PTX moves the membrane potential of red cells toward the equilibrium potentials for Na and K, proving that the PTX-induced cation permeability pathways are electrically conducting. Our results also show that the maximum K and Na fluxes induced by PTX are ~10 mol/liter of cells per h, consistent with the notion that PTX binds to the Na,K pump protein and opens a channel of ~10 pS/pump.

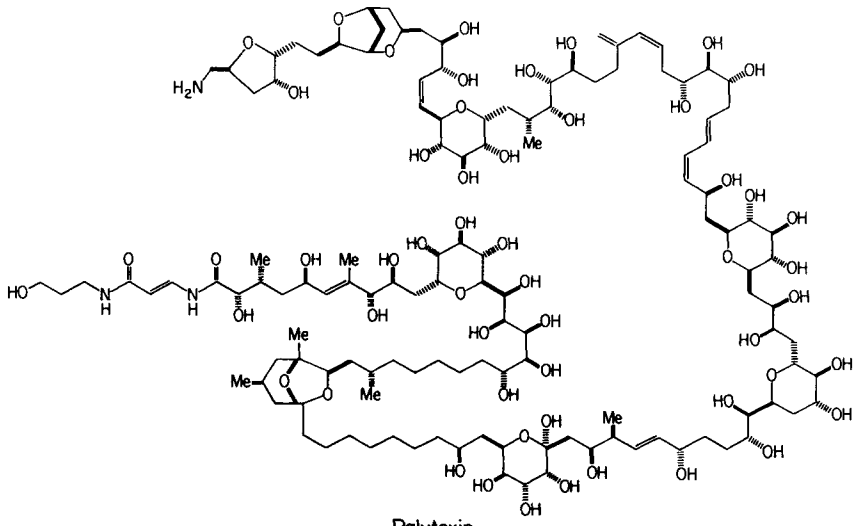
The results reported in this article suggest that PTX blocks the hydrolysis of ATP by the Na,K-ATPase present in the intact red cell membrane. Further, since we also found that both inhibition of hydrolysis and channel formation by PTX are reversed when PTX is removed from the membrane, we conclude that PTX seems to convert the Na,K pump into a Na,K-permeable channel in human red cell membranes in reversible fashion.

We also report that *N*-(*p*-bromobenzoyl)-palytoxin (NPB-PTX), a derivative of PTX in which the terminal nitrogen is blocked, is two to three orders of magnitude less potent than the parent compound in its ability to modify the permeability of the red cell membrane to cations. In addition, we found that NPB-PTX is about two orders of magnitude less effective than PTX in removing ouabain from its binding sites on the red cell membrane. Thus, at least part of the reduced potency of NPB-PTX is due to reduced affinity of this analogue for the ouabain binding site on the Na,K-ATPase.

MATERIALS AND METHODS

All salts used were reagent grade and were purchased from Mallinckrodt Inc. (St. Louis, MO). Carbonylcyanide *m*-chlorophenylhydrazone (CCCP), ouabain, sucrose, nystatin, albumin, vanadate, Triton X-100, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were purchased from Sigma Chemical Co. (St. Louis, MO). DMSO was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Methazolamide (Neptazene) was a gift from Lederle Laboratories (Pearl River, NY).

The PTX (Fig. 1) used in this study was isolated from Okinawan *Palythoa tuberculosa*, and was a generous gift from Profs. K. Hirata and D. Uemura (Nagoya University, Nagoya, Japan). The sample used was free from the minor palytoxins, as well as free of palytoxin carboxylic acid, as judged from the profiles in HPLC and the ^{1}H and ^{13}C NMR spectra, and had a purity better than 98% (HPLC). The analogue, (*p*-bromobenzoyl)-palytoxin was prepared using the procedure described by Uemura, Hirata, Iwashita, and Naoki (1985). The product was examined spectroscopically (1D and 2D ^{1}H NMR and FAB mass spectra) and its purity estimated to be better than 98%. The lyophilized samples were kept at −50°C until ready to be used, when we prepared 3×10^{-4} M stock solutions of both compounds by dissolving them with a 0.1% aqueous solution of albumin. They were then kept at −50°C until needed for further dilutions.



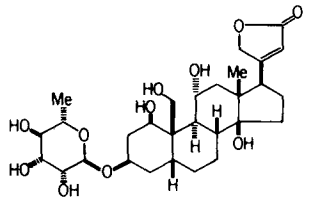


FIGURE 1. Chemical structure of PTX (*top*) according to Uemura et al. (1985), and chemical structure of ouabain (*bottom*).

General

Since the effect of PTX depends on the amount of cells present (at a fixed PTX concentration) we have chosen to indicate the concentration of toxin as the amount of toxin "per liter of cells (l.c.)" rather than "per liter of cell suspension (l)." The various media used in these studies always contained 0.5 mM Na borate, since it has been shown that this concentration is optimal for the effects of PTX on human red cells (Ahnert-Hilger, Chhatwal, Hessler, and Habermann, 1982). All experiments described were performed at 37°C.

Preparation of Red Cells

Human blood was collected in heparinized tubes from healthy donors and washed five times in 150 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Tris-MOPS (pH 7.4 at 0°C). Methods to determine cell Na and K concentration, hemoglobin, and mean corpuscular hemoglobin concentration (MCHC) were as previously described (Brugnara, Kopin, Bunn, and Tosteson, 1985).

Determination of Membrane Potential (V_m) in Erythrocytes

The membrane potential of human red cells was estimated through measurements of the hydrogen ion distribution across the red cell membrane in unbuffered medium containing DIDS and methazolamide, each at a concentration of 10 and 50 μM CCCP (Macey, Adorante, and Orme, 1978; Halperin, Brugnara, Tosteson, van Ha, and Tosteson, 1989). When necessary, the internal anion composition of the cells was changed by preincubating fresh cells at 37°C in the medium of the desired composition for ~30 min. 30 μM DIDS was then added to inhibit further exchange of anions. A portion of the cells was then washed three times in the medium in which the change in the membrane potential was to be measured, always in the presence of DIDS. These packed cells were then added to the prewarmed medium, the pH of which was previously adjusted to 7.4 with a concentrated solution of the hydroxide of the major external cation.

Flux Measurements

Measurement of radioactive fluxes in fresh and nystatin-treated cells. When necessary, the internal cation composition of the cells was changed by treating the cells with the ionophore nystatin. This procedure, as well as the measurements of ^{22}Na influx and ouabain-sensitive ^{86}Rb influx, were performed as previously described (Brugnara et al., 1985).

Measurement of Na efflux from vanadate-treated cells. Human RBC were loaded with vanadate using the high-hematocrit dialysis method as described by Dale (1987). Briefly, the erythrocytes were washed with cold phosphate buffer (150 mM NaCl and 5 mM Na phosphate, pH 6) and then concentrated to a hematocrit of ~90%. A volume of packed cells (5–10 ml) was placed into dialysis tubing (10,000 mol wt cut-off) and the bag was tightly sealed. The dialysis bag was placed in a bottle containing 500 ml of lysis buffer (8 mM NaCl, 2 mM Na_2PO_4, 0.2 mM $MgCl_2$, and 1 mM vanadate, pH 6.5 at 0°C) and the bottle was sealed and rotated in a vertical plane at 6–10 revolutions per min, immersed in a 0°C ice bath. Lysis continued for 3 h, after which the dialysis bag was changed to a bottle containing an isotonic resealing solution (135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 0.1 mM EGTA, 2.4 mM ATP, 2 mM adenine, 2.5 mM inosine, 0.1 mM vanadate, and 10 mM Tris-MOPS, pH 7.4). Resealing proceeded by rotation of the bottle in a 37°C water bath for 1 h and then the resealed cells were washed five times with resealing solution and five times with choline washing solution. At the end of the procedure, the activity of the Na,K pump was assessed through measurements of the ouabain-sensitive ^{86}Rb influx.

The Na efflux into Na-free medium (140 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Tris-MOPS, pH 7.4) was measured by centrifugation of the cell suspension through oil (dibutyl phthalate) as described (Halperin, Brugnara, Kopin, Ingwall, and Tosteson, 1987).

Measurement of K Efflux from Fresh Cells

K efflux from human red blood cells was measured into K-free medium using a K-selective electrode (model 93-19; Orion Research Inc., Cambridge, MA). An aliquot of cells (typically 0.4 ml of an 80% cell suspension) was washed five times in flux medium. The packed cells were then added to 10 ml of medium at 37°C to make a 3% cell suspension under continuous stirring, and the external K was recorded continuously both before and after the addition of PTX (pH meter/datalogger model 6091, with which data points can be recorded every 10 s; Jenco Electronics Ltd., San Diego, CA).

Measurement of Lactate Production and Cell ATP

Measurements of lactate production and cellular ATP in cells exposed to PTX and treated with nystatin to contain 100 mmol/l.c. NaCl and 0–1 mmol/l.c. KCl were performed as previously described (Halperin et al., 1987).

Ouabain Binding Studies

Measurements of the competition of ^{3}H-labeled ouabain by ouabain, PTX, and NPB-PTX were performed as previously described by Halperin et al. (1987), in cells that were made to be nominally K-free (Na substitution) using the nystatin method, in order to avoid displacement of [^{3}H]ouabain and inhibition of PTX binding due to an increase in the extracellular K as a consequence of exposure to PTX.

Washout of Bound PTX

Washout of cells that had been exposed to PTX was done using a modification of the procedure described by Ahnert-Hilger et al. (1982). Cells (3% hct) were incubated for 20 min at 37°C with PTX (2.8 nM) in a medium containing 140 mM NaCl, 0.5 mM Na borate, and 10 mM Tris-MOPS, pH 7.4. At the end of the incubation period the cell suspension was diluted with a solution (PS) containing 140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-MOPS, and 0.1% albumin and spun down. The cells were then washed three times with PS, each time allowing the cells to incubate with the medium for 5 min before spinning the cells down. To determine the ouabain-dependent Rb influx, the cells were further washed two times in the flux medium consisting of 140 mM NaCl, 2 mM KCl, and 10 mM Tris-MOPS.

RESULTS

Cation Permeability Induced by PTX

It has been reported by Weidmann (1977), Muramatsu, Uemura, Fujiwara, and Narahashi (1984), and Castle and Strichartz (1988) that excitable cells depolarize at rest when exposed to PTX. This has been mainly attributed to formation of nonselective cation pores near or within the pump protein. If the same mechanism is operative in nonexcitable tissue such as red blood cells, the membrane potential of these cells should also change upon exposure to PTX. Fig. 2 shows the results of experiments designed to determine the change in the membrane potential of human red cells upon exposure to varying concentrations of PTX. Seen in the figure is the fact that, as reported for other cells (Rouzaire-Dubois and Dubois, 1990), there is a lag between the time of addition of PTX and the beginning of the response. As the concentration of PTX is increased, the time required to reach a plateau is shortened, as is the case with the lag between the time of addition of PTX and the beginning of the response. Shown also in Fig. 2 *A* is the fact that the effect of PTX on membrane potential can be reversed upon addition of ouabain to the medium.

Since, under normal physiological conditions, the conductance of the red cell membrane is dominated by the conductance of the anions, a change in the membrane potential such as that seen upon addition of PTX indicates an increase in the conductance of the membrane for cations. This increase, irrespective of the main anion present (NO_3 or Cl), is a function of [PTX]. Fig. 2 *B* shows that as [PTX]

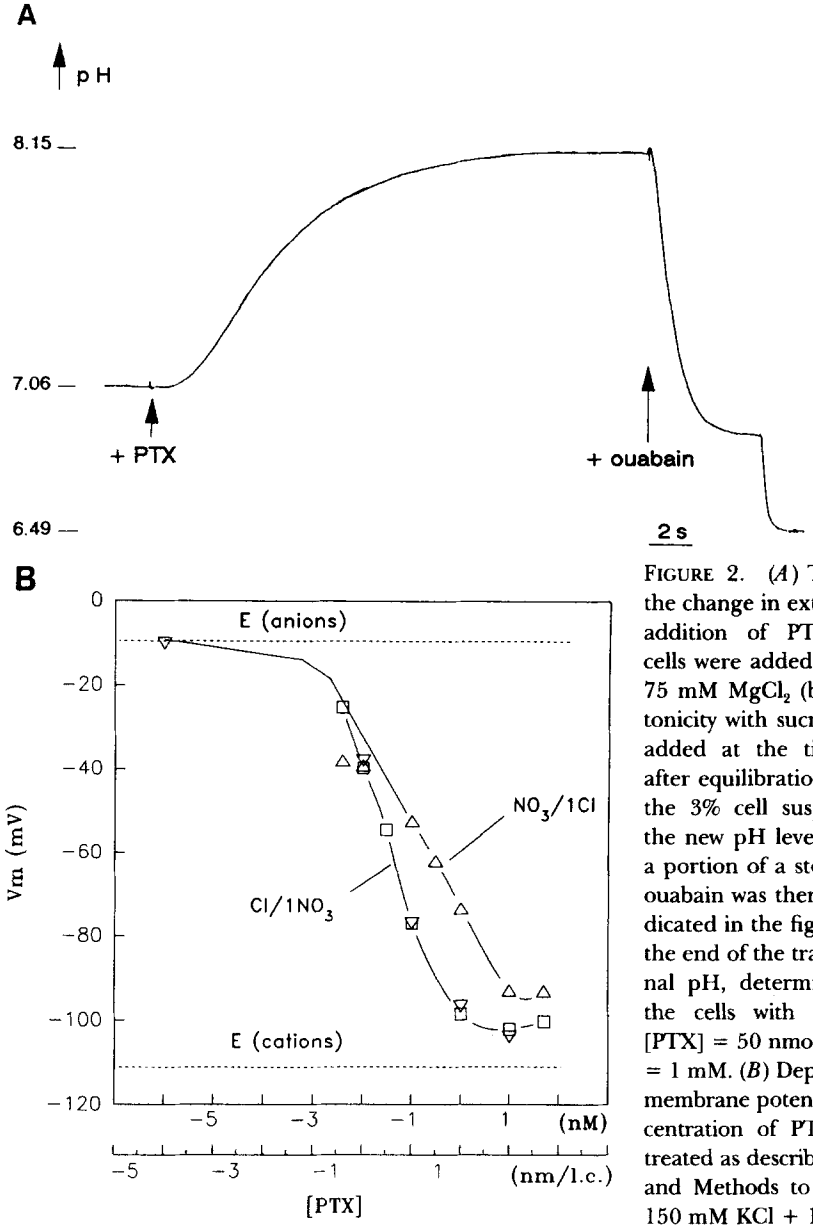


FIGURE 2. (*A*) Time course of the change in external pH after addition of PTX. Untreated cells were added to unbuffered 75 mM $MgCl_2$ (brought to isotonicity with sucrose). PTX was added at the time indicated, after equilibration of the pH of the 3% cell suspension. After the new pH level was attained, a portion of a stock solution of ouabain was then added (as indicated in the figure). Shown at the end of the trace is the internal pH, determined by lysing the cells with Triton X-100. [PTX] = 50 nmol/l.c.; [ouabain] = 1 mM. (*B*) Dependence of the membrane potential on the concentration of PTX. Cells were treated as described in Materials and Methods to contain either 150 mM KCl + 1 mM KNO_3 or 150 mM KNO_3 + 1 mM KCl, and were subsequently used to determine the change in membrane potential upon serial additions of PTX at the concentrations indicated to a medium containing 75 mM Mg, 0.5 mM Na, and 1.5 mM K. The line labeled "E (anions)" represents the membrane potential obtained under the present experimental conditions, in the absence of PTX. The line labeled "E (cations)" is the Nernst potential for the permeant cations (Na + K). The curves are drawn through the points obtained in three separate experiments. Before the measurements, the cells were washed three times in the flux medium containing, in addition to the salt as indicated, DIDS and methazolamide, each at a concentration of 10 and 50 μM CCCP, as described in Materials and Methods. Hematocrit, 3%.

increases, so does the cationic conductance of the red cell membrane, which is converted into a cation-selective membrane at [PTX] >3 nmol/l.c. The increase in the cationic conductance was also seen in the absence of DIDS, but the concentration of PTX required to have the same change in the membrane potential was higher than in the presence of DIDS (data not shown).

To assess the PTX-induced increase in the permeability of the red cell membrane to cations other than K, we determined the change in membrane potential when PTX is present in media in which the main cation was varied, as shown in Fig. 3. The permeability to cations relative to that of K was calculated using the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949), in which the

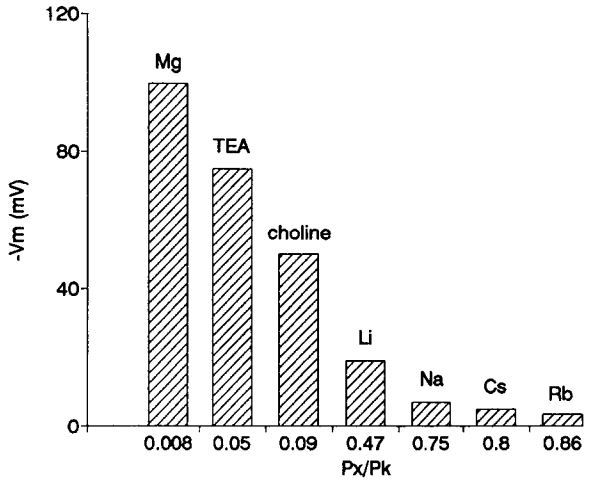


FIGURE 3. Effect of varying the major external cation on the PTX-induced membrane potential of red cell membranes. Untreated cells were washed with a solution containing the major cation as indicated in the figure, as well as DIDS, methazolamide, and CCCP. The packed cells were then added to a prewarmed, unbuffered medium of the same composition, to make a 3% cell suspension. After equilibration of the external pH, a portion of a stock solution of PTX was added to a final concentration of 10 nmol/l.c. and the pH was determined again at the steady state. The permeability ratio was calculated as indicated in the text.

permeability to anions was assumed to be negligible, since the PTX concentration used was >3 nmol/l.c. (cf. Fig. 2 *B*).

$$V_m = \frac{R \times T}{F} \times \log \frac{P_X \times [X]_o + P_K \times [K]_o}{P_X \times [X]_i + P_K \times [K]_i}$$

where R is the gas constant, F the Faraday number, T the temperature, $[X]$ the ion concentration (i, inside, and o, outside the cell), and the P's are the permeability to cations.

Dependence of Cation Fluxes on [PTX]

From the data described in Figs. 1 and 2, it is evident that the rate of movement of cations across the red cell membrane must be increased by several orders of magnitude as the PTX concentration is increased. To quantitate this effect, we measured K efflux into a K-free, Na medium. Fig. 4 shows the time course of the PTX-induced K efflux from human red cells. As shown in Fig. 2, Fig. 4 also illustrates that ouabain prevents the further loss of K, even in the continued presence of PTX.

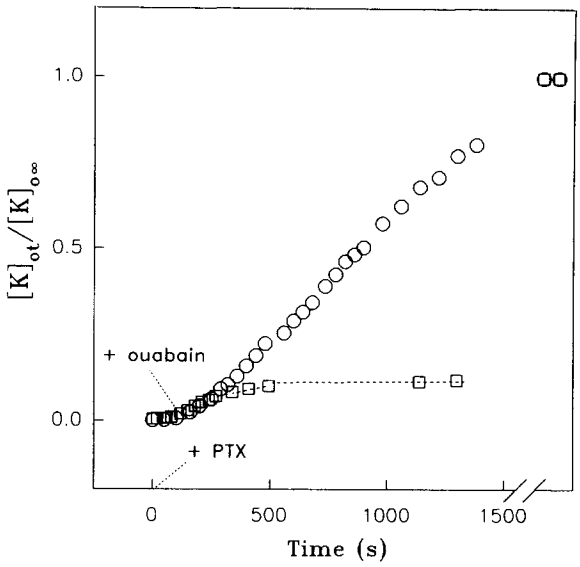


FIGURE 4. Time course of the PTX-induced movement of K down its electrochemical potential gradient. Fresh cells were added to a prewarmed, K-free medium (140 mM NaCl and 10 mM Tris-MOPS, pH 7.4 at 37°C) to have a 3% cell suspension and the [K] in the medium ($[K]_{ot}$) was measured continuously with the K-selective electrode for 5–10 min. PTX was then added to a final concentration of 6 nmol/l.c. In one set (○) the cells were allowed to continue until all the K had leaked out of the cells, as judged by no further change in the [K] in the medium ($[K]_{o\infty}$) upon addition of Triton X-100 to the suspension. In a second set (□), ouabain (100 μM final concentration) was added at the time indicated and the K efflux continued to be monitored. The K remaining in these cells was determined by addition of Triton X-100 to the suspension 30 min after addition of ouabain.

As [PTX] is increased, the K efflux is increased, as illustrated in Fig. 5, which shows that the shape of the curve relating the K flux to [PTX] is biphasic, suggesting that PTX binds to a site with two different affinities or to two different, noninteracting sites, one with low capacity and high affinity and the other with high capacity and low

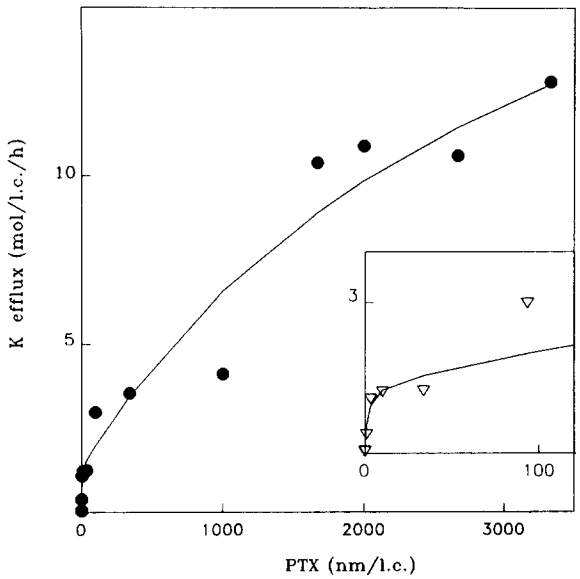


FIGURE 5. Effect of varying the concentration of PTX on the K efflux. Experimental conditions were as described in Fig. 4. The line through the points is the result of a least-squares curve fit to the data, assuming that there are two independent binding sites (K_{a1} = 0.05 nM, V_{max1} = 1.4M/l.c. per h, and K_{a2} = 98 nM, V_{max2} = 24 mol/l.c. per h). The inset shows the points at low [PTX].

affinity. The data at present do not allow us to distinguish between these two possibilities. The best fit to the data was obtained assuming that there are two independent binding sites with apparent dissociation constants K_1 = 0.05 nM and K_2 = 99 nM, and corresponding maximum fluxes v_{m1} = 1.4 mol/l.c. per h and v_{m2} = 24 mol/l.c. per h. K_1 is of the same order of magnitude found by Böttinger, Béress, and Habermann (1986) for the binding constant of ^{125}I-labeled PTX (0.02 nM) in the presence of Ca^{2+}. Rouzaire-Dubois and Dubois (1990) have modeled their results of the effect of PTX on the steady-state current across neuroblastoma cell membranes as produced by the formation of a channel from the binding of at least two PTX molecules to a membrane receptor, with dissociation constants of 0.001 and 5 nM. We have not been able to fit our data to such a model.

Dependence of Cation Fluxes on Time of Exposure to PTX

Since it has recently been reported that the effect of PTX on neuroblastoma cells is transient (Rouzaire-Dubois and Dubois, 1990), resembling the ion permeability

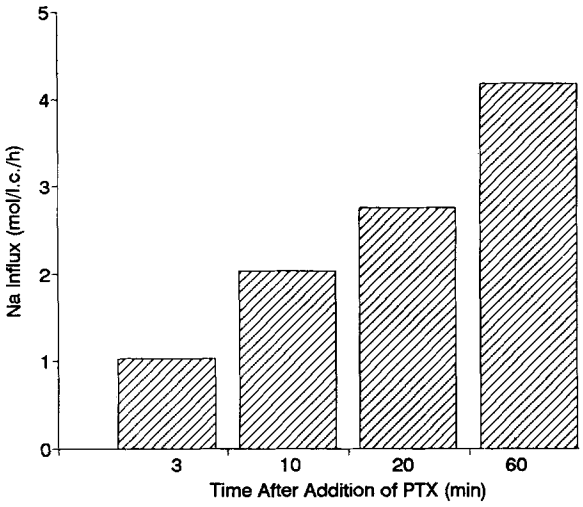


FIGURE 6. Time dependence of the effect of PTX. The Na influx was determined as indicated in Materials and Methods, using cells that had been pretreated with nystatin to be nominally K-free ($[Na^+]_i$ = 100 mmol/l.c.; $[K^+]_i \leq$ 0.5 mmol/l.c.). The tracer was added at the times indicated and samples were taken 1 and 3 min after, spun through dibutyl phthalate, and used to estimate the ^{22}Na uptake. [PTX] = 50 nmol/l.c.

pathways produced in red cell membranes by other pore formers such as melittin (Tosteson, Holmes, Razin, and Tosteson, 1985) or complement (Halperin, Nicholson-Weller, Brugnara, and Tosteson, 1988), we decided to study the effect that PTX has on the Na permeability of human red cells as a function of the time the cells had been exposed to PTX. As shown in Fig. 6, the increase in the cation permeability of red cells is not a transient phenomenon, but rather it continues to increase with increasing time of exposure to the toxin.

Effects of pH on the PTX-induced Potassium Fluxes

During the determination of the change in membrane potential induced by PTX, measurements of the pH of the interior of the cells at the end of the experiments, after exposure of the cells to PTX, showed a significant acidification of the cells' interior, as has been reported by Frelin, Vigne, and Breittmayer (1990) to occur in

chick cardiac cells. Given the buffer capacity of hemoglobin, the observed decrease in the internal pH corresponds to the addition of ~30 mmol of protons/l.c. To determine if this intracellular acidification is due to K/H exchange, we measured the K fluxes into media of varying pH. The results of these experiments, depicted in Table I, indicate that a K/H exchange cannot be assumed to occur when human red cells are exposed to PTX, since the fluxes were higher the lower the H^+ concentration in the external medium. Note also that the fluxes in the presence of Mg^{2+} as the major external cation are lower than the fluxes measured when Na^+ is the major external cation. The difference between the K efflux into Mg-containing medium and the efflux into Na-containing medium can be accounted for if the movement of K induced by PTX is primarily via electro-chemical diffusion, taking into account that the membrane potential is around −100 mV in the Mg medium and −6 mV in the Na medium.

TABLE I

Effect of pH on the PTX-induced K Flux

Conditions	K efflux		
	pH 6.4	*mmol/l.c. per h* pH 7.4	pH 8.4
$MgCl_2$ + PTX (154 nmol/l.c.)	370	740	900
NaCl + PTX (176 nmol/l.c.)	2,470	3,170	4,490

The K fluxes were measured in untreated fresh cells using the K-sensitive electrode, as indicated in Materials and Methods, in three different experiments. The cells were kept at 4°C at a hematocrit of ~70%. At the time of the experiment, an aliquot (0.5 ml) was withdrawn and washed three times in the flux medium (at room temperature). The packed cells were then added to 10 ml prewarmed medium and the efflux of K was followed for 5–10 min before addition of an aliquot of PTX to have the desired final concentration.

Effect of Vanadate on the Increase in Cation Permeability Induced by PTX

It has been shown that the effects of PTX on K permeability are inhibited by prior exposure of cells to ouabain (Habermann and Chhatwal, 1982), presumably because the receptor site for PTX is then occupied. To determine if the effects of PTX are also antagonized by inhibitors of the pump acting at sites other than the ouabain-binding site, cells were pretreated to contain vanadate before exposure to PTX. We chose vanadate because it acts on the inside of the cells (Cantley, Josephson, Warner, Yanagisawa, Lechene, and Guidotti, 1977), and it has also been shown by Myers, Boerth, and Post (1979) to enhance the binding of ouabain to the Na,K-ATPase. The results of these experiments, shown in Table II, indicate that vanadate, like ouabain, antagonizes the effect of PTX on the cation fluxes, suggesting that the vanadate form of the enzyme either does not bind PTX or that PTX bound to this form is unable to open the permeability pathway that characterizes its action. These results are in contrast to those obtained by Chhatwal, Hessler, and Habermann (1983), who did not find any change in the sensitivity of red cell ghosts to PTX upon incorporation of vanadate.

TABLE II

Effect of Vanadate on the Ouabain-sensitive, PTX-induced Na Efflux

Conditions	K influx			Na efflux
	Total	+ Ouabain	Ouabain-sensitive	Total
	mmol/l.c. per h			
Control	2.5	0.1	2.4	8.6
+ PTX (35 nmol/l.c.)				97.0
Vanadate-treated	0.3	0.2	0.1	6.7
+ PTX (35 nmol/l.c.)				9.8

The internal content of the cells was changed to have cells containing Na as the major internal cation, using the dialysis technique detailed in Materials and Methods. A portion of the cells was also exposed to 1 mM vanadate during the lysis period. The K influx was measured using ^{86}Rb (see Materials and Methods for details). The Na efflux was measured into a Na-free medium containing 140 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM MOPS-Tris, pH 7.4. Results from one of two experiments.

Effects of PTX on the Na Pump of Intact Red Cells

Since the magnitude of the cationic fluxes produced in the presence of PTX are several orders of magnitude greater than the ouabain-inhibited fluxes of Na and K through the Na,K pump, to estimate the extent of inhibition of the Na pump of the red cell membrane by PTX we measured the ATP consumption and lactate production when the toxin was added to a suspension of human erythrocytes. The results of the experiments on the effects of PTX on the Na,K pump are depicted in Table III. The table shows that when red cells are exposed to a concentration of PTX greater than the total number of pumps, both the ouabain-sensitive lactate production and the ATP hydrolysis are inhibited, suggesting that the Na,K-ATPase has been inhibited by PTX. Both of the effects of PTX, channel formation and inhibition of

TABLE III

Effect of PTX on the ATP Hydrolysis and Lactate Production of Human Red Cells

Conditions	Na efflux	Lactate production	[ATP]	
			0 min	90 min
	mmol/l.c. per h		*mmol/l.c.*	
Control	8.63	3.0	0.94	0.32
+ Ouabain	2.6	1.0	1.00	0.97
+ PTX (2.3 nmol/l.c.)	166	2.25		
+ Ouabain	NM	1.97		
+ PTX (23 nmol/l.c.)	480	1.7		
+ Ouabain	NM	1.7		
+ PTX (56 nmol/l.c.)			0.98	0.68
+ Ouabain			0.94	0.84

Cells were pretreated with nystatin to have 100 mmol/l.c. NaCl and 1 mmol/l.c. KCl, as described in Materials and Methods. A portion of the cells was used to determine the pump fluxes and the PTX-stimulated Na efflux. Two other aliquots were used for the determination of lactate production and ATP consumption, following the protocols described in Materials and Methods. Results from one of two experiments which yielded similar results. NM, not measured.

TABLE IV

Effect of PTX and N-blocked PTX on the Permeability of the Red Cells to K

	K efflux	
[Compound]	PTX-induced	(NPB-PTX)-induced
nmol/l.c.	*mmol/l.c. per h*	
0	2.5	2.5
0.04	40	—
16.7	—	10
33.3	1,204	20
2,000	10,900	170

The K fluxes were measured in untreated fresh cells, with the K-sensitive electrode as described in Materials and Methods. The values were obtained in at least five different experiments.

ATP hydrolysis, can be substantially reversed by thorough washing of the cells that had been exposed to PTX (cf. Table V).

Consequences of Blocking the NH_2 Terminal of PTX

The working hypothesis of the action of PTX on cells is that this compound binds to the ouabain-binding site on the Na,K-ATPase and produces an increase in the cation permeability either by further interaction with the enzyme or through interactions at another site in the vicinity of the enzyme. It has been speculated, on stereochemical grounds, that the COOH-terminal end is the region of the PTX molecule that binds to the ouabain-binding site and that the NH_2-terminal end plays a role in the increase in the cationic permeability of cells (cf. Fig. 1). To test this hypothesis, we have characterized the actions of an analogue of PTX (NPB-PTX) in which the NH_2-terminal end was blocked as described in Materials and Methods. Table IV shows that NPB-PTX is two to three orders of magnitude less potent than PTX in its ability to induce an increase in the permeability to K. This result is consistent with the findings of Ohizumi and Shibata (1980), who observed that *N*-acetylpalytoxin is

TABLE V

Effect of N-blocked PTX on the Ouabain-sensitive K Influx and Reversal of the Effect of PTX on the Pump Fluxes

	K influx		
Additions	Total	+ Ouabain	Ouabain-sensitive
	mmol/l.c. per h		
None	1.84	0.44	1.40
NPB-PTX (26.3 nm/l.c.)	2.40	0.37	2.03
NPB-PTX (270.0 nm/l.c.)	3.17	0.51	2.66
NPB-PTX (2,326.0 nm/l.c.)	4.09	0.60	3.49
Washed PTX (93.0 nm/l.c.)	2.64	0.58	2.06

The K fluxes (control and in the presence of NPB-PTX) were determined on fresh red cells and the values are representative of three different experiments. "Washed PTX" corresponds to cells that had been preexposed to 93 nm/l.c. PTX and then carefully washed to remove the toxin, as described in Materials and Methods.

1/100 as potent as PTX in its excitatory action in the isolated guinea pig vas deferens, suggesting that the lower potency of NPB-PTX is probably due to a lack of free NH_2-terminal end. It is of interest to note that the action of NPB-PTX does not require the presence of borate, suggesting that borate binds at or near the NH_2-terminal end of PTX and that this binding might be impeded by the bulky benzoyl derivative.

Since the permeability to cations is barely increased, it is possible to measure the ouabain-sensitive K influx in cells exposed to NPB-PTX. As illustrated in Table V, there is no inhibition of the pump flux in the presence of the analogue, even when the number of NPB-PTX molecules was as high as 400 times that of the number of pumps. The increase in the ouabain-sensitive flux observed in the table can be attributed to an increase in the passive permeability to cations induced by NPB-PTX

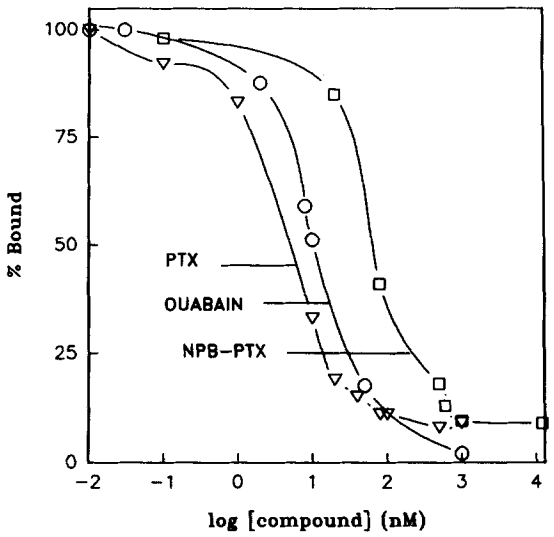


FIGURE 7. Competition of [^{3}H]ouabain (○), PTX (▽), and NPB-PTX (□) for binding sites on the human erythrocyte membrane. The cells were added to a medium containing [^{3}H]ouabain (4 nM) together with the desired concentrations of unlabeled ouabain or toxins, and incubated for 5 h at 37°C. The results were calculated in cycles per minute of [^{3}H]ouabain bound per milliliter of cell and expressed as the ratio to the amount bound in the absence of added compounds. The lines were drawn through the points.

(antagonized by ouabain), as is evident from the increase in the total K influx shown in the table (cf. also Table IV).

To determine if the failure of NPB-PTX to induce an increase in the permeability to cations and to block the pump is due to a lack of binding of the compound to the ouabain binding site on the red cell membrane, we studied the competition of [^{3}H]ouabain binding by NPB-PTX. The results of these studies are illustrated in Fig. 7, which shows that NPB-PTX competes with ouabain for its site, but with an affinity which is >10 times lower than PTX. These results, together with those of Ohizumi and Shibata (1980), then suggest that the NH_2-terminal end of PTX plays a role both in binding and in the establishment of the permeability pathway induced by PTX.

DISCUSSION

In the light cast by the observations described in this communication, several interesting questions about the action of PTX on membranes containing the Na,K

pump come to mind. First, where in the tertiary and quaternary structures of this or other proteins are the PTX-induced channels located? Second, are the channels unique to membranes containing PTX, or does the toxin inhibit closure of channels that are normally opening and closing too rapidly to observe? Third, is there a naturally occurring PTX that opens physiologically significant channels in or near Na,K pumps in the membranes of excitable cells? Fourth, what is the relation of the actions of PTX on the Na,K pump and its demonstrated effects on the arachidonic acid cascade and osteoclast activity? And, fifth, in addition to the free terminal nitrogen, how much of the rest of the PTX molecule is required for binding to the Na,K pump and for the permeability pathway to open (or to remain opened).

With regard to the locus of the PTX-induced channels, it is possible that they go through the Na,K pump. However, it is also possible that they involve the border lipid and/or the PTX itself. In this regard, it is interesting to note that Yoda, Rosner, Morrison, and Yoda (1990) have reported a PTX-induced increase in Na influx (in the presence of CCCP, A23187, and [PTX] = 10 pM) which is partially inhibited by ouabain in their liposomes containing right-side-out Na,K-ATPase (electric organ, electric eel). Rey, Meda, and Anner (1988) have found a partial inhibition of the ouabain-dependent K influx into liposomes containing right-side-out Na,K-ATPase (rabbit kidney) in the presence of relatively high concentrations of PTX (10–100 μM). We have looked carefully for increases in cation permeability with vesicles containing the Na,K-ATPase purified from shark rectal gland and failed to find them. We did observe PTX inhibition of both Na pumping and ATPase activity in such vesicles and PTX-induced increase of Na and K permeability in shark red cells at relatively low concentrations of PTX (Tosteson, M. T., and F. Cornelius, unpublished observations). It is possible that some component of the Na,K pump complex required for channel formation by PTX is missing in the vesicles containing the enzyme purified from shark rectal gland, or in the medium in which the assays were done. It is also possible that the sensitivity of the pump protein to PTX is species dependent. Clearly, more work in this area is needed to resolve these issues.

In resolving the issue of the molecular locus of the PTX-induced channels, it is important to identify the form of the Na,K pump with which PTX reacts. In this regard, the evidence is compelling that PTX behaves like ouabain and reacts with a phosphorylated intermediate of the Na,K-ATPase, E_2P (for a review of the kinetic scheme of the pump enzyme, see Cantley, 1981). The inhibition of PTX action in red cell membranes by vanadate reported here lends further support to that conclusion.

The fact that all of the Na,K pumps in the red cell membrane can be converted to open channels by PTX, and can then be restored to normal activity by removal of the toxin, raises the question of whether such channels are part of the normal mode of operation of the pump (cf. Läuger, 1979). If so, the duration of the open times for the channels must be short enough so that they do not contribute appreciably to the membrane conductance, nor permit internal shunting of the pump. However, it is not unreasonable to speculate that there may be brief times when the pumping mechanism itself is moving an "occluded" ion from one side of the membrane to the other when there exists a pathway for ions to move all the way across the membrane. In such a case, the role of PTX could be to inhibit the rapid closure of such normally occurring but extremely short-lived channels.

It is noteworthy that not all of the actions of PTX on cells can be easily explained by its capacity to convert Na,K pumps into Na,K leaks. Lazzaro, Tashjien, Fujiki, and Levine (1987) have reported that PTX is an extremely potent agonist of the arachidonic acid cascade in osteoclasts. A consequence of this action is increased bone resorption activity by these cells. These effects of PTX are completely prevented by ouabain and therefore presumably a consequence of the binding of PTX to the Na,K pump and the opening of channels. How these several events are connected will provide new insights into the regulation of the arachidonic acid cascade and bone metabolism. A plausible hypothesis worth testing is that the change in membrane potential produced by the opening of PTX-induced channels is the connecting event.

The most remarkable feature of the action of PTX on cell membranes is that it competes with ouabain for binding to the Na,K pump and that, like ouabain, it inhibits the Na,K-ATPase (cf. Fig. 7 and Table II), but unlike ouabain, PTX opens cation-permeable channels. It will be interesting and important to determine the molecular basis for this spectrum of action. A first step in this direction is to note the effect of blocking the terminal nitrogen of PTX, reported in this paper. This modification is sufficient to reduce by an order of magnitude the affinity of PTX for the ouabain binding site on the Na,K pump (cf. Fig. 7) and to reduce by two to three orders of magnitude both its capacity to inhibit Na,K-ATPase activity and to open channels. We intend to search for derivatives of PTX that bind as tightly to the Na,K pump as the parent compound but do not produce channels, in an effort to separate, if possible, these two actions. Further, we will work with derivatives of PTX designed to answer the question of which are the necessary (and sufficient) parts of the molecule to produce its effects.

We are very grateful to Dr. C. Brugnara for his help with some of the experiments reported.

Original version received 4 February 1991 and accepted version received 8 July 1991.

REFERENCES

Ahnert-Hilger, G., G. S. Chhatwal, H.-J. Hessler, and E. Habermann. 1982. Changes in erythrocyte permeability due to palytoxin as compared to amphotericin B. *Biochimica et Biophysica Acta.* 688:486–494.

Böttinger, H., L. Béress, and E. Habermann. 1986. Involvement of (Na + K)-ATPase in binding and actions of palytoxin on human erythrocytes. *Biochimica et Biophysica Acta.* 861:165–176.

Brugnara, C., A. S. Kopin, H. F. Bunn, and D. C. Tosteson. 1985. Regulation of cation content and cell volume in patients with homozygous hemoglobin C disease. *Journal of Clinical Investigation.* 75:1608–1617.

Cantley, L. C. 1981. Structure and mechanism of the (Na,K)-ATPase. *Current Topics in Bioenergetics.* 11:201–237.

Cantley, L. C., L. Josephson, R. Warner, M. Yanagisawa, C. Lechene, and G. Guidotti. 1977. Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. *Journal of Biological Chemistry.* 252:7421–7423.

Castle, N. A., and G. R. Strichartz. 1988. Palytoxin induces a relatively non-selective cation permeability in frog sciatic nerve which can be inhibited by cardiac glycosides. *Toxicon.* 26:941–951.

Chhatwal, G. S., H.-J. Hessler, and E. Habermann. 1983. The action of palytoxin on erythrocytes and resealed ghosts: formation of small, nonselective pores linked with Na,K-ATPase. *Naunyn-Schmiedeberg's Archives of Pharmacology.* 323:261–268.

Dale, G. L. 1987. High efficiency entrapment of enzymes in resealed red cell ghosts by dialysis. *Methods in Enzymology.* 149:229–234.

Frelin, C., P. Vigne, and J.-P. Breittmayer. 1990. Palytoxin acidifies chick cardiac cells and activates the Na/H antiporter. *FEBS Letters.* 264:63–66.

Goldman, D. E. 1943. Potential, impedance and rectification in membranes. *Journal of General Physiology.* 27:37–60.

Grell, E., E. Lewitzi, and D. Uemura. 1988. Interaction between palytoxin and purified Na,K-ATPase. *Progress in Clinical and Biological Research.* 268B:393–400.

Habermann, E. 1989. Palytoxin acts through Na,K-ATPase. *Toxicon.* 27:1171–1187.

Habermann, E., and G. S. Chhatwal. 1982. Ouabain inhibits the increase due to palytoxin of cation permeability of erythrocytes. *Naunyn-Schmiedeberg's Archives of Pharmacology.* 319:101–107.

Habermann, E., G. Ahnert-Hilger, G. S. Chhatwal, and L. Béress. 1981. Delayed hemolytic action of palytoxin: general characteristics. *Biochimica et Biophysica Acta.* 649:481–486.

Halperin, J. A., C. Brugnara, A. S. Kopin, J. Ingwall, and D. C. Tosteson. 1987. Properties of the Na-K pump in human red cells with increased number of pump sites. *Journal of Clinical Investigation.* 80:128–137.

Halperin, J. A., C. Brugnara, M. T. Tosteson, T. van Ha, and D. C. Tosteson. 1989. Voltage-activated cation transport in human erythrocytes. *American Journal of Physiology.* 26:C986–C996.

Halperin, J. A., A. Nicholson-Weller, C. Brugnara, and D. C. Tosteson. 1988. Complement induces a transient increase in membrane permeability in unlysed erythrocytes. *Journal of Clinical Investigation.* 82:594–600.

Hodgkin, A. L., and B. Katz. 1949. Effect of Na on the electrical activity of the giant axon of the squid. *Journal of Physiology.* 108:61–88.

Ishida, Y., K. Takagi, M. Takahashi, N. Satake, and S. Shibata. 1983. Palytoxin isolated from marine coelenterates. The inhibitory action on (Na,K)-ATPase. *Journal of Biological Chemistry.* 258:7900–7902.

Läuger, P. 1979. A channel mechanism for electrogenic ion pumps. *Biochimica et Biophysica Acta.* 552:143–161.

Lazzaro, M., A. H. Tashjien, H. Fujiki, and L. Levine. 1987. Palytoxin: an extraordinarily potent stimulator of prostaglandin production and bone resorption in cultured mouse calvariae. *Endocrinology.* 120:1338–1345.

Macey, R. I., J. S. Adorante, and F. W. Orme. 1978. Erythrocyte membrane potentials determined by hydrogen ion distribution. *Biochimica et Biophysica Acta.* 512:284–295.

Moore, R. E., and P. J. Scheuer. 1971. Palytoxin: a new marine toxin from coelenterates. *Science.* 172:495–498.

Muramatsu, I., D. Uemura, M. Fujiwara, and T. Narahashi. 1984. Characteristics of palytoxin-induced depolarization in squid axons. *Journal of Pharmacology and Experimental Therapeutics.* 231:488–494.

Myers, T. D., R. C. Boerth, and R. L. Post. 1979. Effects of vanadate on ouabain binding and inhibition of (Na+K)-ATPase. *Biochimica et Biophysica Acta.* 558:99–107.

Ohizumi, Y., and S. Shibata. 1980. Mechanism of the excitatory action of palytoxin and N-acetylpalytoxin in the isolated guinea-pig vas deferens. *The Journal of Pharmacology and Experimental Therapeutics.* 214:209–214.

Ozaki, H., H. Nagase, and N. Urakawa. 1985. Interaction of palytoxin and cardiac glycosides on erythrocyte membrane and (Na+K)ATPase. *European Journal of Biochemistry.* 152:475–480.

Rey, H. G., P. Meda, and B. M. Anner. 1988. Two-sided functional Na,K-ATPase-liposomes for characterizing the permeability and side of action of pump inhibitors. *Progress in Clinical and Biological Research.* 268B:429–436.

Rouzaire-Dubois, B., and J.-M. Dubois. 1990. Characterization of palytoxin-induced channels in mouse neuroblastoma cells. *Toxicon.* 28:1147–1158.

Tosteson, M. T., S. J. Holmes, M. Razin, and D. C. Tosteson. 1985. Melittin lysis of red cells. *Journal of Membrane Biology.* 87:35–44.

Uemura, D., Y. Hirata, T. Iwashita, and H. Naoki. 1985. Studies on palytoxins. *Tetrahedron.* 41:1007–1017.

Weidmann, S. 1977. Effects of palytoxin on the electrical activity of dog and rabbit heart. *Experientia.* 33:1487–1489.

Yoda, A., M. R. Rosner, P. Morrison, and S. Yoda. 1990. Interaction of Na,K-ATPase proteoliposomes with palytoxin at pM concentrations. *The Journal of General Physiology.* 96:74*a*. (Abstr.)