Large Diameter of Palytoxin-induced Na/K Pump Channels and Modulation of Palytoxin Interaction by Na/K Pump Ligands

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ABSTRACT Palytoxin binds to Na/K pumps to generate nonselective cation channels whose pore likely comprises at least part of the pump’s ion translocation pathway. We systematically analyzed palytoxin’s interactions with native human Na/K pumps in outside-out patches from HEK293 cells over a broad range of ionic and nucleotide conditions, and with or without cardiotonic steroids. With 5 mM internal (pipette) [MgATP], palytoxin activated the conductance with an apparent affinity that was highest for Na⁺-containing (K⁺-free) external and internal solutions, lowest for K⁺-containing (Na⁺-free) external and internal solutions, and intermediate for the mixed external Na⁺/internal K⁺, and external K⁺/internal Na⁺ conditions; with Na⁺ solutions and MgATP, the mean dwell time of palytoxin on the Na/K pump was about one day. With Na⁺ solutions, the apparent affinity for palytoxin action was low after equilibration of patches with nucleotide-free pipette solution. That apparent affinity was increased in two phases as the equilibrating [MgATP] was raised over the submicromolar, and submillimolar, ranges, but was increased by pipette MgAMPPNP in a single phase, over the submillimolar range; the apparent affinity at saturating [MgAMPPNP] remained ~30-fold lower than at saturating [MgATP]. After palytoxin washout, the conductance decay that reflects palytoxin unbinding was accelerated by cardiotonic steroid. When Na/K pumps were preincubated with cardiotonic steroid, subsequent activation of palytoxin-induced conductance was greatly slowed, even after washout of the cardiotonic steroid, but activation could still be accelerated by increasing palytoxin concentration. These results indicate that palytoxin and a cardiotonic steroid can simultaneously occupy the same Na/K pump, each destabilizing the other. The palytoxin-induced channels were permeable to several large organic cations, including N-methyl-d-glucamine⁺, suggesting that the narrowest section of the pore must be ~7.5 Å wide. Enhanced understanding of palytoxin action now allows its use for examining the structures and mechanisms of the gates that occlude/deocclude transported ions during the normal Na/K pump cycle.

KEY WORDS: Na/K-ATPase • ion-motive pump • ion channel selectivity • outside-out patch recording • toxin binding

INTRODUCTION

The lethal marine toxin, palytoxin, first extracted from the polyps of soft-bodied zoanthids of the genus _Palythoa_ (Moore and Scheuer, 1971), is a potentially useful tool for elucidating the molecular reactions by which the Na/K-ATPase stoichiometrically transports Na⁺ and K⁺ ions in opposite directions across the cell surface membrane. Palytoxin was found to depolarize mammalian cells (e.g., Weidmann, 1977) by causing small-conductance (~10 pS) relatively nonselective cation channels to appear in their surface membranes (e.g., Ikeda et al., 1988; Muramatsu et al., 1988). That the Na/K-ATPase is the target for palytoxin action was initially surmised (Habermann, 1989) largely from antagonism of toxin action by cardiotonic steroids, like ouabain, or by K⁺ agonism of toxin action by cardiotonic steroids, like ouabain-sensitive cation flux in yeast expressing both α and β subunits of mammalian Na/K pumps, but not if the yeast expressed either subunit alone (Scheiner-Bobis et al., 1994), nor if the COOH-terminal 44 residues of the α subunit were deleted (Redondo et al., 1996). Moreover, because binding of [³H]ouabain to intact yeast cells, and its inhibition by palytoxin, established that the truncated Na/K-ATPase was expressed at the cell surface and could interact with either ligand, it seemed that palytoxin somehow opened a cation pathway within, or alongside, intact Na/K pumps. But catalytic integrity was not essential, as palytoxin still elicited ouabain-sensitive cation efflux in yeast expressing mutant Na/K pumps in which the α subunit’s phosphate-accepting residue, Asp369, was replaced by Ala (Scheiner-Bobis and Schneider, 1997). The second approach used mammalian αβ Na/K pumps synthesized by in vitro translation in a cell-free expression system and then incorporated into a planar phospholipid bilayer, where exposure to palytoxin elicited the characteristic ~10-pS cation channels (Hirsh and Wu, 1997), so dem-
onstrating unequivocally that Na/K pumps are the sole requirement for palytoxin action.

More recent results have suggested that the palytoxin-induced pores comprise at least some segment of the ion translocation pathway normally traversed by the pumped Na\(^+\) and K\(^+\) ions. Thus, palytoxin has been shown to render cysteines substituted for several residues in the fifth and sixth putative transmembrane helices (M5 and M6) susceptible to hydrophilic sulfhydryl-specific reagents (Guennoun and Horisberger, 2000, 2002). Functional consequences of mutating some of those residues had already implicated them in cation binding (e.g., Nielsen et al., 1998; Pedersen et al., 1998), and their homologues in the related SERCA-ATPase were found, in high-resolution crystal structures (Toyoshima et al., 2000; Toyoshima and Nomura, 2002), to help coordinate the bound Ca ions. In addition, ion flow through individual palytoxin-induced channels appeared to be controlled by two gates that could be regulated by the Na/K pump's physiological ligands in a way that suggested that gating reflects conformational changes like those underlying the pump's normal ion occlusion/deocclusion partial reactions (Artigas and Gadsby, 2003a,b). Together, these studies suggest that palytoxin interferes with the normally strict coupling between inner and outer sets of gates that control access to cation binding sites within the Na/K pump, and thereby allows the gates to sometimes be open simultaneously. If so, then palytoxin could be a useful tool for characterizing the ion translocation pathway and the structures and mechanisms of the pump’s gates.

Before palytoxin can be exploited for such studies, however, its interactions with the Na/K pump must be better understood. Despite much work (for review see Habermann, 1989; Tosteson, 2000), a consistent picture of how palytoxin interacts with the Na/K pump has yet to emerge. This is undoubtedly due to the variety of methods, preparations, and Na/K pump isoforms examined, and the limited range of experimental conditions employed in each study (Tosteson, 2000), as there were early indications that the effects of palytoxin could be influenced by, among other things, extracellular K ions (Ahnert-Hilger et al., 1982; Ishida et al., 1983; Böttinger et al., 1986) or cytoplasmic nucleotides (Chhatwal et al., 1983; Böttinger et al., 1986; Kim et al., 1995). Here we describe an extensive study of palytoxin interactions with native Na/K pumps in HEK293 cells (a human embryonic kidney cell line) and in guinea pig ventricular myocytes. Almost all measurements were made on outside-out excised patches of membrane so that the compositions of solutions at both surfaces could be well controlled. We found that the apparent affinity for the interaction of palytoxin with the same population of Na/K pumps was profoundly influenced by varying the ionic and metabolic conditions. Palytoxin appeared to bind most tightly to phosphorylated Na/K pumps exposed to K\(^+-\)-free, Na\(^+-\)-containing external and internal solutions: interestingly, these conditions, combined with physiological cytoplasmic [MgATP], correspond to those found to maximize the open probability of palytoxin-induced channels (Artigas and Gadsby, 2003a). We found that palytoxin and a cardiotonic steroid can both be simultaneously bound to the same Na/K pump, but that each destabilizes the binding of the other. We also present a preliminary characterization of the permeation pathway of the palytoxin-induced channel which suggests that, at its narrowest point, the pore must have a diameter on the order of 7.5 Å.

**MATERIALS AND METHODS**

**Cell Preparation**

Human embryonic kidney (HEK293) cells were maintained at 37°C and 5% CO\(_2\), in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (all from Gibco-BRL), and then plated on polyl-lysine-coated glass coverslips in 35-mm Petri dishes for use within 3 d. Guinea pig ventricular myocytes were isolated using collagenase as described (Gadsby and Nakao, 1989), and stored until use (within 24 h) at 4°C in high-K Ca-free solution containing (in mM) 70 K-glutamate, 25 KCl, 20 taurine, 10 KH\(_2\)PO\(_4\), 1 MgCl\(_2\), 0.5 EGTA, 20 glucose, 10 HEPES (pH = 7.3).

**Solutions**

For whole-cell and outside-out patch recording, the standard cytoplasmic-side (pipette) solution contained (in mM): 130 t-glutamic acid, 10 HEPES, 10 TEA\(_2\)-lysine–glutamic acid, 10 MgATP, 1 MgCl\(_2\), and 150 NaOH or KOH (or, to measure Na/K pump current, 50 NaOH + 100 CO\(_3\)\(_2\)). Tetrathylenammonium (TEA) was omitted from the internal solutions containing 100 mM tetraptopylammonium (TPA) or N-methyl-D-glucamine (NMG). The external solution contained (in mM): 140 sulfamic acid, 10 HEPES, 10 HCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 0.5 BaCl\(_2\), and 160 mM of the appropriate monovalent cation (as indicated). For whole-cell measurements of Na/K pump current 1 mM GdCl\(_3\) was added to minimize currents through Na\(^+\) and Ca\(^2+\) channels and the Na/Ca exchanger. MgATP was omitted from nucleotide-free internal solution and intermediate [MgATP] obtained by mixing. When the nucleotides Li\(_2\)AMPPNP (5’-adenylylimido-diphosphate) or Na\(_2\)ADP (adenosine 5’-diphosphate) replaced MgATP, equimolar MgCl\(_2\) was added to keep free [Mg\(^{2+}\)] ~1 mM. All internal and external solutions had a pH of 7.4 ± 0.05 and osmolality of 280–305 mosM/kg.

An aliquot of frozen 100 μM PTX (from *Palythoa toxica*; Calbiochem) aqueous solution was thawed and diluted into external solutions just before each experiment. All PTX-containing solutions also included ~0.002% BSA to reduce PTX binding to nonglass surfaces (Taylor et al., 1991). Strophanthidine was added from a 1-M stock solution in DMSO, and ouabain and dihydro-ouabain were dissolved in the appropriate external solution at their final concentration.

**Electrophysiology**

Whole-cell and outside-out membrane patch currents were recorded (Hamill et al., 1981) at 22–25°C using an Axopatch 200B
of outside-out patches of membrane excised from HEK293 cells. The prolonged recording in Fig. 1 A shows the large inward shift of membrane current caused by addition of 100 nM PTX, a saturating concentration (Fig. 4, below), to the 160-mM Na⁺ solution bathing a patch held at −20 mV with 150 mM Na⁺ and
5 mM MgATP in the intracellular (pipette) solution. The inward current represents inflow of Na\(^{+}\) ions, under the influence of the \(\sim 20\)-mV inward driving force, through the thousands of opened channels resulting from PTX action on every Na/K pump in the membrane patch; maximal PTX-induced current in 12 patches under these conditions was 930 \(\pm\) 170 pA, indicating an average of \(\sim 7,000\) pump channels per patch, given their open probability of \(\sim 0.9\) and single-channel conductance of \(\sim 7.5\) pS (Fig. 3, below). The time course of current increase was approximately exponential under these conditions, with time constant \(\tau_{\text{inc}} = 9 \pm 2\) s \((n = 7)\). Despite washout of all unbound toxin a few seconds later, the current returned to practically the same level during each of the subsequent five reexposures to Na-containing solution, suggesting that PTX remained bound for many minutes, implying a low rate of toxin unbinding (see also Fig. 4 A, below).

We examined selectivity of PTX-induced channels by measuring near steady-state current-voltage relationships in the presence of various external cations (with constant 150 mM Na\(^{+}\) in the pipette), both before and after opening the channels with PTX. The ramp-like current deflections during the exposure to PTX (Fig. 1 A, ***) indicate large changes in current elicited by 80-ms steps to membrane potentials from \(-150\) to \(+90\) mV, reflecting the increased conductance of the membrane caused by PTX. The barely discernible current deflections in Na\(^{+}\) solution (Fig. 1 A, **) before addition of PTX confirm that membrane conductance was initially low. Examples of these current changes are shown on a greatly expanded time-scale in Fig. 1 B. Current levels near the end of every step before PTX were subtracted from those after exposure to PTX to yield the PTX-induced currents \(I_{\text{PTX}}\) shown in the current-voltage plot labeled “Na\(^{+}\)” (Fig. 1 C, ●). The PTX-induced current reversed sign near 0 mV, as expected for current carried by the principal cation, Na\(^{+}\), present at roughly the same concentration on both sides of the membrane.

Switches between the Na\(^{+}\) external solution and solutions containing instead 160 mM NMG\(^{+}\), TMA\(^{+}\), or TEA\(^{+}\) before PTX caused only negligibly small changes in holding current, and current deflections in response to the voltage steps were also small (e.g., NMG\(^{+}\), Fig. 1 B). But, after PTX exposure, the voltage-elicted current changes became considerably larger in all these solutions, and replacing Na\(^{+}\) by any of these cations (or by Tris\(^{+}\), or arginine\(^{+}\)) caused large outward shifts of holding current (Fig. 1 A), indicating that they were all less permeant than Na\(^{+}\) through the PTX-induced channels. PTX-induced current-voltage plots show that for each of these organic cations the outward current shift reflected a negative shift of the reversal potential (e.g., from +5 mV in Na\(^{+}\) to \(-90\) mV in NMG\(^{+}\), Fig. 1 C). On the assumption that external solution exchange was quick enough to allow internal [Na\(^{+}\)] to remain constant, the observed shift in reversal potential for each cation (relative to Na\(^{+}\)) was used to calculate the ratio of permeability coefficients, \(P_{X}/P_{Na}\) (see MATERIALS AND METHODS): e.g., \(P_{\text{NMG}}/P_{\text{Na}}\) averaged 0.021 \(\pm\) 0.002 \((n = 11)\). Plotting these permeability ratios against the mean diameters (geometric mean of three dimensions of the smallest box to contain a space-filling model) of the cations (dehydrated for the organic cations, hydrated for Na\(^{+}\); Robinson and Stokes, 1965; Villarroel et al., 1995) suggests that permeability falls to near zero when the mean diameter of the cation is \(\sim 7\) Å (Fig. 1 D). Indeed, an estimate of 7.5 Å for the diameter of the narrowest part of the PTX-induced channel pore was obtained by fitting to the data (Fig. 1 D, curve) a simplified model (e.g., Dwyer et al., 1980) in which the cross-sectional area available to permeating ions determines the permeability of a rigid cylindrical water-filled pore.

To verify that the small inward PTX-induced currents at very negative potentials in external NMG\(^{+}\) solution truly reflected inflow of NMG\(^{+}\) ions, and not of Na\(^{+}\) ions that had exited the pipette through the PTX-opened channels and accumulated in some poorly-stirred external layer, we repeated the measurements after replacing all Na\(^{+}\) in the pipette solution with large monovalent cations (Fig. 2). With 160 mM pipette NMG\(^{+}\) (and 5 mM MgATP, as usual), addition of 100 nM PTX to the 160-mM NMG\(^{+}\)-containing external solution caused an inward shift of membrane current (at the \(-20\)-mV holding potential), with a time course (\(\tau_{\text{inc}} = 15 \pm 6\) s, \(n = 6)\) comparable to that seen with all-Na\(^{+}\) solutions, but with an amplitude smaller by one or two orders of magnitude (Fig. 2 A, left). As found with Na\(^{+}\)-containing external solution, despite washout of unbound toxin, the inward current during the final exposure to NMG\(^{+}\) solution was unchanged (Fig. 2 A, left), suggesting that PTX also dissociated slowly under these experimental conditions. In addition, the voltage-induced current deflections as well as the shifts of membrane current caused by switching between external solutions containing 160 mM NMG\(^{+}\) or 160 mM TPA\(^{+}\), or by diluting the NMG\(^{+}\) solution \(\sim 10\)-fold (15 mM NMG\(^{+}\)) with isotonic sucrose, were all much larger after PTX exposure than before. The corresponding PTX-induced current-voltage plots (Fig. 2 A, right), all obtained with 160 mM internal NMG\(^{+}\), confirm that NMG\(^{+}\) ions do indeed permeate PTX-induced channels in both directions. Moreover, the negative reversal potential shift due to the 10-fold reduction in [NMG\(^{+}\)] averaged \(-60 \pm 5\) mV \((n = 3)\), close to the \(-59\)-mV estimated shift in equilibrium potential for NMG\(^{+}\) ions. This suggests that the simultaneous 10-fold reduction in [sulfamate\(^{-}\)] was of little consequence, and hence
that the PTX-induced channels are essentially impermeable to these large anions.

In contrast, only very small PTX-induced currents were seen in the external solution containing 160 mM TPA$^+$ (Fig. 2 A, right). To examine this further, we used 160 mM TPA$^+$ internal solution and repeated the experiment (Fig. 2 B). Under these conditions, little or no PTX-induced current was discernible when 160 mM TPA$^+$ was present also in the external solution, and there was no response to an ~10-fold dilution of the external TPA$^+$ solution with isotonic sucrose solution (Fig. 2 B). However, after washout of unbound PTX, a switch to NMG$^+$ external solution elicited a rapid inward current shift and revealed a PTX-induced inward current-voltage relationship (Fig. 2 B, right) comparable to that observed under the conditions of Fig. 2 A. As the PTX had already been washed out, the fact that the inward current was activated more rapidly in Fig. 2 B than in Fig. 2 A suggests that PTX had in fact bound to the Na/K pumps in TPA$^+$ external solution, and transformed them into ion channels, but that TPA$^+$ was too large a cation to permeate them. This conclusion is supported by the evident inability of the 160 mM internal TPA$^+$ to carry any outward PTX-induced current at large positive membrane potentials (even during exposure to NMG$^+$ external solution, when substantial inward currents were seen at negative potentials; Fig. 2 B, right). Indeed, TPA$^+$ appeared to exert some blocking effect as outward currents carried by NMG$^+$ were reduced, reversibly, during exposure to TPA$^+$ external solution (Fig. 2 A). These results establish that the ion pathway underlying the PTX-induced conductance is wide enough to allow permeation of NMG$^+$ (mean diameter, 7.3 Å), but narrow enough to preclude permeation of TPA$^+$ (mean diameter, 8–9 Å).

Experiments like that in Fig. 1 (using the same Na$^+$-containing internal solution) yielded permeability ratios, relative to Na$^+$, for other alkali metal cations: $P_{K}/P_{Na} = 1.13 \pm 0.02$ ($n = 6$), $P_{Ca}/P_{Na} = 1.01 \pm 0.01$ ($n = 3$), and $P_{Rb}/P_{Na} = 1.11 \pm 0.02$ ($n = 2$) (Artigas and Gadsby, 2003b). These ratios confirm earlier reports that the PTX-induced conductance pathway selects poorly among monovalent inorganic cations (e.g., Ikeda et al., 1988; Muramatsu et al., 1988; Tosteson et al., 1991; for review see Tosteson, 2000). In agreement with findings on neuroblastoma cells (Rouzaire-Dubois and Dubois, 1990) and red blood cells (Tosteson et al., 1991), we also found the divalent cations Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ to be weakly (≤NMG$^+$), but demonstrably, permeant through PTX-induced channels: e.g., holding current of HEK293 cells (held at −20 mV, with NMG$^+$ internal solution) shifted inward on addition of 100 nM PTX to isotonic Ca$^{2+}$ solutions devoid of monovalent cations (not depicted). The low permeability explains previous failure to observe PTX-induced Ca$^{2+}$ currents in single-channel recordings (Ikeda et al., 1988).

Single-channel Characteristics of PTX-induced Channels

To confirm that the large PTX-induced currents in HEK293 cells (Fig. 1) and cardiac myocytes (Fig. 10, below) flow through channels with characteristics like those previously reported (e.g., Muramatsu et al., 1988; Ikeda et al., 1988; Kim et al., 1995), we recorded currents in individual PTX-induced channels. The high density of Na/K pumps (>1,000 μm$^{-2}$) in both cell types, and their high apparent affinity for PTX, make

![Figure 2. NMG$^+$, but not TPA$^+$, ions permeate PTX-induced pathway.](image-url)
were interrupted by brief intraburst closures (e.g., Fig. 3 A, asterisks) and that were separated by somewhat longer interburst shut times. Recordings at different membrane potentials (e.g., Fig. 3 B) yielded single-channel current-voltage plots (e.g., Fig. 3 C) displaying weak inward rectification. Linear fits over the negative voltage range (e.g., line, Fig. 3 C) gave single-channel conductances that averaged $7.1 \pm 0.8 \, \text{pS} \, (n = 4)$ for patches from myocytes, and $7.7 \pm 0.3 \, \text{pS} \, (n = 4)$ for patches from HEK293 cells (two with 5 mM, and two with $<1 \, \mu\text{M MgATP in the pipette}$).

Consistent with the macroscopic permeability measurements, the conductance of single PTX-induced channels ($\text{Na}^+$ internal solution with or without ATP) with $\text{Cs}^+$ as the external cation was $1.15 \pm 0.02 \, (n = 2)$ -fold, and with external $\text{K}^+$ was $1.22 \pm 0.15 \, (n = 4)$ -fold, that with $\text{Na}^+$ internal and external solutions.

### Interaction of PTX with Na/K Pumps

To establish a basis for understanding the variability in published apparent affinities for PTX interaction with the Na/K pump (for review see Tosteson, 2000), we systematically examined those interactions using the same preparation, outside-out patches excised from HEK293 cells, but over a wide range of ionic and nucleotide conditions.

#### Interactions in Symmetrical (Internal and External) High [Na$^+$] or High [K$^+$] Solutions

We began with 5 mM MgATP and 150 mM Na$^+$ in the pipette (cytoplasmic-side solution), and 160 mM external Na$^+$ solution. Because the dissociation rate of PTX is extremely low under those conditions (e.g., Fig. 1 A), we attempted to determine cumulative [PTX]-response curves by applying progressively increasing concentrations of PTX and waiting for the resulting current increment to approach steady-state. At the lowest concentrations tested, 8 and 10 pM PTX, the slow current increase necessitated exposures lasting up to 45 min (Fig. 4 A). For each [PTX], the time course of the increase in current could be reasonably approximated by a single exponential function (e.g., Fig. 4 A, dotted fit lines) from which the rates could be estimated and steady-state current increments extrapolated; the fits at 8 and 10 pM in Fig. 4 A yielded $\tau_{inc} = 1,971 \, \text{s}$ and $1,442 \, \text{s}$, respectively. The switch to 1 nM PTX produced a much faster current increase ($\tau_{inc} = 210 \, \text{s}$; Fig. 4 A) to a near maximal level, as there was little further response to 10 or 100 nM PTX; in other patches, these higher concentrations were applied alone to obtain corresponding $\tau_{inc}$ estimates. After withdrawal of the toxin, the PTX-induced current decayed extremely slowly (note the 2.5-fold contraction of time scale in Fig. 4 A); assuming eventual return to the original current baseline, an exponential fit (fit line largely obscured by data, Fig. 4 A)

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**Figure 3.** Small-conductance channels underlie PTX-induced current. (A, top left) Absence of channel activity in an outside-out ventricular-myocyte patch held at $-40 \, \text{mV}$, with external and internal Na$^+$ solutions and 5 mM internal MgATP, before PTX application. (Right) A single PTX-induced channel opened $\sim$1 min after application of 20 pM PTX and its activity, characterized by long open bursts with brief closures (e.g., asterisks), continued long after PTX washout. (Bottom) Examples of brief intraburst closures (asterisks, above) shown on expanded time scale; no baseline subtraction was used. (B) Records of current through same channel (asterisks, above) shown on expanded time scale; no baseline subtraction until a channel appeared (after 1 min in the case of the myocyte patch in Fig. 3 A), whereupon unbound PTX was promptly washed away. Because PTX unbinds slowly under these conditions (e.g., Figs. 1 and 4), the channel continued gating for many minutes, with a high open probability ($P_o = 0.93 \pm 0.02$, $n = 3$; Artigas and Gadsby, 2003a), due to prolonged open bursts that

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suggests $\tau_{\text{dec}} \sim 10^5$ s (fits to somewhat shorter current decays after PTX washout in two other patches gave estimates for $\tau_{\text{dec}}$ of 2 $\times$ 10^4 s and 5 $\times$ 10^4 s).

Up to 500 nM PTX, the conductance activation rate $1/\tau_{\text{inc}}$ increased linearly with [PTX] (Fig. 4 B, fit line), consistent with the relationship $1/\tau_{\text{inc}} = k_{\text{on}}[\text{PTX}] + k_{\text{off}}^*$ (in which $k_{\text{off}}^* = 1/\tau_{\text{dec}}$ is negligibly small, as observed), which suggests that PTX binding is the rate-limiting step for channel opening under these conditions: the fit gave $k_{\text{on}} = 1.4 \pm 0.1 \times 10^6$ M$^{-1}$s$^{-1}$. The [PTX] dependence of the steady-state level of PTX-induced current, $I_{\text{PTX}}$, extrapolated for low [PTX] and normalized to the current at 10 nM, was reasonably well described by a Michaelis-Menten function (Fig. 4 C, fit line), with $K_{\text{m,PTX}} = 33 \pm 7$ pM.

For comparison, we repeated these measurements after replacing all Na$^+$ with K$^+$; i.e., we used 160 mM external K$^+$, and 150 mM K$^+$ and 5 mM MgATP in the pi-
pette. Two major consequences were immediately evident (Fig. 5 A): activation of measurable current required ~1,000-fold higher concentrations of PTX, and the rate of conductance activation remained low even at the highest PTX concentrations. That this slower activation is attributable to the substitution of extracellular K⁺ for Na⁺ ions is directly confirmed by the much faster activation of PTX-induced current when 50 nM PTX was applied later to the same patch after switching to Na⁺-containing external solution (Fig. 5 A, continuation of recording in lower panel).

The fact that washout of PTX had led to the recovery of baseline conductance, allowing that subsequent reapplication of PTX, points to another distinction between extracellular Na⁺ and K⁺ ions. Namely, after withdrawal of PTX (after equilibration with 500 nM PTX) the current decay was extremely slow in the presence of external Na⁺ (τdec ~6,000 s; Fig. 5 A, dashed fit line) but was accelerated >10-fold upon switching to external K⁺ (τdec ~500 s, dotted fit line; mean 1/τdec = 3 ± 1 × 10⁻³ s⁻¹, n = 4). This relatively rapid current return to near the starting level, and full reinstatement of maximal PTX-induced current on reapplication of PTX, confirms that the current decay reflected unbinding of PTX. The sudden increase in current upon switching from Na⁺ to K⁺ solution is accounted for by the ~15% higher permeability of PTX-induced channels for K⁺ than for Na⁺ ions described above.

The saturating dependence of the activation rate on [PTX] (Fig. 5 B) implies that a slow step, distinct from PTX binding to the pump, rate-limits activation of the conductance at high [PTX] in K⁺ solutions. The influence of [PTX] on steady-state current (normalized to that at 500 nM) was again reasonably approximated by Michaelis kinetics with kₘ,PTX = 22 ± 2 nM (Fig. 5 C). This ~1,000-fold lower apparent affinity for PTX in all-K⁺ solutions than found with all-Na⁺ solutions seems only partly attributable to the observed ~100-fold increase in the rate of current decline that likely reflects PTX unbinding (compare Figs. 4 A and 5 A) in all-K⁺ solutions.

**External K⁺ Is Responsible for the Low Rate of Conductance Activation at Saturating [PTX]**

To further examine which of the two ions, on which side of the membrane, was responsible for these differences, we repeated the PTX concentration-response measurements after changing the ion on only one side of the membrane at a time, i.e., with Na⁺ outside and K⁺ inside (Naₒ/Kᵢ; Fig. 6, A and B), or with K⁺ outside and Na⁺ inside (Kₒ/Naᵢ; Fig. 6, C and D). When PTX was applied in 160 mM external Na⁺ solution with 150 mM K⁺ and 5 mM MgATP in the internal solution, the activation rate of the PTX-induced current showed no sign of saturation up to 100 nM [PTX] (Fig. 6 A) and the linear fit to 1/τinc = kₘ[PTX] + k⁰off gave kₘ = 8.3 ± 0.3 × 10⁻⁵ M⁻¹s⁻¹, using the single estimate of k⁰off (= 1/τdec = 10⁻⁴ s⁻¹) obtained directly from current decay after PTX washout; this kₘ is comparable to the value we obtained with all-Na⁺ solutions. However, the apparent affinity for activation of steady-state current by PTX in this mixed Naₒ/Kᵢ condition, which yielded Kₘ,PTX = 0.27 ± 0.07 nM (Fig. 6 B), was intermediate between that found in all-K⁺ or all-Na⁺ solutions (10-fold lower affinity than in all-Na⁺).

In the complementary biionic condition, with 160 mM external K⁺ solution and with 150 mM Na⁺ and 5 mM MgATP in the internal solution, the rate of activation of the PTX-induced current remained low as [PTX] was increased and it saturated at high [PTX] (e.g., ≥50 nM; Fig. 6 C), similar to our findings with all-K⁺ solutions. The current decay rate, related to PTX...
unbinding, in this mixed K_0/Na condition was 1/τ_{dec} = 9 ± 3 × 10^{-4} \text{s}^{-1} (n = 7), some threefold slower than with all-K^+ but ~30-fold faster than with all-Na^+ solutions. Similarly, PTX increased steady-state current in the mixed K_0/Na condition with an apparent affinity (K_{0.5PTX} = 2.4 ± 0.8 \text{nM}; Fig. 6 D) intermediate between those found in all-K^+ or all-Na^+ solutions (100-fold lower affinity than in all-Na^+). The faster (compared with external Na^+) current decay after PTX washout elicited by external K^+ was also observed with external Cs^+, Rb^+, or Tl^+ solutions but not with external solutions of the organic cations NMG^+ or TMA^+; and qualitatively similar results were obtained whether the internal solution contained Na^+ or K^+

Evidently, regardless of whether the internal solution contained K^+ or Na^+, the presence of 160 mM external K^+ severely limited the rate of activation of PTX-induced current at maximal [PTX] (Figs. 5 B and 6 C), and (compared with external Na^+) increased K_{0.5PTX} ~100-fold (Figs. 5 C and 6 D vs. Figs. 6 B and 4 C). This uniformity of external cation effects, together with the consistent ~10-fold increase of K_{0.5PTX} caused by replacing internal Na^+ with K^+, regardless of external cation (Figs. 4 C and 6 D vs. Figs. 6 B and 5 C), mitigates against major distortion of these results by intrappetite accumulation (estimated to be ≤8 mM; see Kang et al., 2003) of ions flowing through opened channels at the ~20-mV holding potential. An important mechanistic question is whether this influence of extracellular K^+ is mediated by K^+ ions binding to their transport sites in the Na/K pump. If so, the rate of current activation at high [PTX] may be expected to display a dependence on external [K^+] comparable to that found for K^+ transport by the Na/K pump. Indeed, with internal solution containing 150 mM Na^+ and 5 mM MgATP, current activation by 100 nM PTX was slowed by external [K^+] (replacing extracellular Na^+; Fig. 7 A), with K_{0.5K} = 2.4 ± 1.2 mM (Fig. 7 B), near values for Na/K pump current stimulation by external K^+, in the presence of extracellular Na^+, in HEK293 cells (1.5 mM; Kock-sämer et al., 1997) and other mammalian cell types (e.g., Gadsby and Nakao, 1989; Bielen et al., 1991; Kinnard et al., 1994; Peluffo et al., 2000). Also consistent with this effect being mediated via the external transport sites, the K^+ congeners Cs^+, Rb^+, and Tl^+, all slowed activation of PTX-induced current in side-by-side comparisons with Na^+- or NMG^+-containing external solutions (Fig. 7 C). Notably, in the extreme case of Tl^+ (present as 145 mM TlNO_3), 100 nM PTX appeared incapable of activating any current, although 500 nM PTX was able to very slowly (τ_{dec} > 2,000 s) elicit the conductance (not depicted). This inhibitory influence cannot be attributed to the NO_3^- ions, as addition of 100 nM PTX in external solution containing principally NaNO_3 rapidly activated the conductance, with a

rate of 1/τ_{inc} = 0.07 ± 0.03 s^{-1} (n = 2), similar to that in the usual Na-sulfamate solution.

Influence of Nucleotides on PTX–Pump Interactions

One of the differences between the experimental conditions described above with Na^+-containing internal solution, and those with exclusively K^+ in the internal solution, is that only in the former case is it certain that the Na/K pumps would be phosphorylated by the included MgATP. With that in mind, we examined PTX interactions with the Na/K pump in HEK293 cell patches bathed in all-Na^+ solutions, but either with nucleotides omitted or with 2 mM MgAMPPNP in the pipette, for comparison with the data already obtained using 5 mM MgATP. (We took care to allow ≥10 min equilibration with these pipette solutions, interspersed

Figure 7. Slow activation of I_{PTX} with K^+ external solution is due to K^+ binding at its transport sites. (A) Superimposed records of normalized I_{PTX} activated by 100 nM PTX in outside-out patches (at ~20 mV, with 150 mM Na^+ and 5 mM MgATP in the pipette), showing slowing of activation by increasing external [K^+] (with [K^+]+[Na^+] = 160 mM). (B) Mean (±SEM, of n measurements) 1/τ_{inc} for 100 nM PTX plotted against external [K^+]; red line shows Michaelis fit yielding K_{0.5K} = 2.4 ± 1.2 mM, 1/τ_{inc}(max) = 0.16 ± 0.03 s^{-1}, and 1/τ_{inc}(min) = 0.007 ± 0.003 s^{-1}. (C) Mean (±SEM, of n measurements) rates of I_{PTX} activation by 100 nM PTX with indicated ionic conditions (external cation/internal cation): K^*/K^+, 0.019 ± 0.004 s^{-1}; Cs^*/K^+, 0.009 ± 0.001 s^{-1}; Rb^*/K^+, 0.008 ± 0.0004 s^{-1}; Tl^*/K^+, 0 s^{-1}; K^*/Na^+, 0.018± 0.004 s^{-1}; Na^*/K^+, 0.084 ± 0.034 s^{-1}; Na^*/Na^+, 0.15 ± 0.03 s^{-1}; NMG^+/NMG^+, 0.12 ± 0.03 s^{-1}. 

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with frequent exposures to external K⁺ solution, to ensure that any initially phosphorylated Na/K pumps would become dephosphorylated. Upon exposure to increasing concentrations of PTX in the absence of pipette nucleotides, 1 nM PTX activated very little current (Fig. 8 A), in contrast to the almost maximal effect obtained with 1 nM PTX in the presence of 5 mM MgATP (compare Fig. 4, above); in fact, with no nucleotides, maximal current activation required ~1 μM PTX. Indeed, Michaelis-Menten fits to the mean data in the absence of nucleotides (Fig. 8 C, blue circles) yielded $K_{0.5PTX} = 19 ± 3$ nM ($n = 6$), confirming that the apparent affinity for PTX was reduced ~1,000-fold compared with 5 mM MgATP (Fig. 8 C, black line, $K_{0.5PTX} = 33$ pM; data from Fig. 4 C, above).

To address whether this reduced affinity might be attributable to lack of phosphorylation, we repeated the measurements with pipette solution containing 2 mM MgAMPPNP (Fig. 8 B), a poorly hydrolyzable MgATP analogue that is essentially incapable of phosphorylating the Na/K pump (ouabain-sensitive Na- and Mg-dependent hydrolysis of AMPPNP is <1% of that of ATP at 20°C; Schuurmans-Stekhoven et al., 1983), but that readily binds to the pump and supports K/K exchange (Simons, 1975). With AMPPNP in the pipette, 1 nM PTX activated a substantial, but still not maximal, current (Fig. 8 B). The dependence on [PTX] of the mean steady-state amplitude of PTX-induced current obtained with 2 mM MgAMPPNP (Fig. 8 C, red triangles) gave $K_{0.5PTX} = 0.76 ± 0.07$ nM ($n = 2$). In a single experiment with 2 mM MgADP in the pipette $K_{0.5PTX} = 0.33$ nM (not depicted), comparable to the value obtained with MgAMPPNP.

The greatly reduced apparent affinity in the absence of nucleotide seems due both to a reduced rate of current activation (Fig. 8 D) and to an increased rate of current decline after withdrawal of the toxin that reflects its unbinding ($\tau_{\text{dec}} = 1,330 ± 260$ s, $n = 7$, estimated from washout periods >400 s; e.g., Artigas and Gadsby, 2003a). This ~10-fold faster current decay than seen with MgATP in the pipette ($\tau_{\text{dec}} > 2 \times 10^4$ s; e.g., Fig. 4 A, above) was similarly found for PTX washout in the presence of MgAMPPNP ($\tau_{\text{dec}} = 2,740 ± 1,990$ s, $n = 4$; estimated as in the absence of nucleotide). Interestingly, however, though current activation by high [PTX] was slowed (~10-fold, Fig. 8 D) by the absence of nucleotide, it was just as rapid with MgAMPPNP in the pipette as with MgATP.

The clear separation of the three curves in Fig. 8 C (5 mM MgATP, 2 mM MgAMPPNP, and no nucleotides) suggests that the influence of [MgATP] on PTX affinity might be seen over two distinct ranges of nucleotide concentration, the submicromolar range that supports pump phosphorylation (Post et al., 1965) and the submillimolar range that promotes ion deocclusion (Furbush, 1987a). To investigate that possibility, $K_{0.5PTX}$ was determined using cumulative dose-response curves (as in Figs. 4 A, 5 A, and 8, A and B) at each of several concentrations of pipette nucleotide, either MgATP or MgAMPPNP. The summarized data (Fig. 9) confirm that $K_{0.5PTX}$ did indeed show a biphasic dependence on cytoplasmic [MgATP] (filled circles) that could be described empirically as the sum of two Michaelis equa-
Interaction between PTX and Cardiotonic Steroids

Previous reports have noted that, in several preparations, preincubation of cells with a cardiotonic steroid, like ouabain, could preclude an increase in membrane permeability when a low concentration of PTX was subsequently applied in the continued presence of the steroid, even though that [PTX] was sufficient to measur-
ditions. However, with no steroid preincubation, conductance activation was faster still in these solutions, and was complete within 10–20 s for 100 nM (e.g., Fig. 10) and within 2 s for 1 μM PTX (compare 500 nM, Fig. 4 B) PTX. The far slower conductance increases, both with 100 nM and with 1 μM PTX, after preincubation to form the cardiotoxic steroid–pump complex, confirm that dissociation of that complex rate limits the increase in conductance. But the more rapid conductance increase elicited by 1 μM than by 100 nM PTX indicates that binding of PTX accelerates dissociation of strophanthidin from its complex with the Na/K pump, and this could occur only if PTX and strophanthidin can simultaneously interact with the same Na/K pump.

The implied reduced affinity for strophanthidin when the Na/K pump is occupied by PTX means that, because of constraints imposed by microscopic reversibility, the apparent affinity for PTX ought to be similarly reduced when the Na/K pump is occupied by strophanthidin. The record in Fig. 11 C shows that after maximal activation of conductance by 100 nM PTX, the current decayed negligibly slowly during ~20 min of toxin washout (compare Fig. 4 A, above), but decayed more rapidly upon exposure to 1 mM ouabain; the same result was obtained with 1 mM dihydroouabain or 0.5 mM strophanthidin (not depicted). Because subsequent withdrawal of ouabain (or strophanthidin) did not restore inward current unless PTX was applied again, the steroid must have accelerated unbinding of PTX rather than merely diminishing PTX-induced conductance (e.g., by closing PTX-opened pump channels; see Artigas and Gadsby, 2003a). Following the above reasoning, this result indicates that cardiotoxic steroids must be able to bind to PTX-modified Na/K pumps. In addition, the slow speed of this action of 1 mM ouabain (τ = 7700 s; Fig. 11 C), a concentration that abolishes Na/K-pump function in seconds (e.g., Fig. 12), further demonstrates the reduced affinity for cardiotoxic steroids of a PTX-modified Na/K pump.

Our conclusion, that the time course of conductance activation by PTX after preincubation with cardiotoxic steroid (Fig. 11, A and B) reflects accelerated steroid unbinding due to binding of PTX, implies that dissociation of the steroid in the absence of PTX should be measurably slower. The time course of Na/K pump current recovery upon washout of cardiotoxin steroid provides a reasonable estimate of steroid unbinding rate in the absence of PTX. Because the small amplitude of Na/K

![Figure 10](https://example.com/figure10.png) Preincubation with a cardiotoxic steroid (CS) slows subsequent activation of I_{PTX} by 100 nM PTX after washout of the steroid. Superimposed records of normalized (to the maximum from exponential fits) I_{PTX} at −20 mV in outside-out HEK293 cell patches with Na⁺ external and internal solutions and 5 mM internal MgATP; note logarithmic time scale. PTX was applied after 5-min preincubations with 1 mM ouabain (red trace, τ_m = 7250 s), or 0.5 mM strophanthidin (blue trace, τ_m = 240 s), or 1 mM dihydroouabain (DHO, green trace, τ_m = 257 s), or with no CS (black trace, τ_m = 6 s).

![Figure 11](https://example.com/figure11.png) PTX and ouabain can simultaneously occupy the same Na/K pump in HEK293 cells. (A) Superimposed representative normalized records of I_{PTX} activation at −20 mV in outside-out HEK293 cell patches with Na⁺ external and internal solutions and 5 mM internal MgATP, after 5-min preincubation with 0.5 mM strophanthidin. I_{PTX} increased more slowly with 100 nM PTX (thin trace) than with 1 μM PTX (thick trace). (B) Bar graph summarizing several (n) measurements: mean τ_m was 256 ± 53 s with 100 nM PTX and 41 ± 7 s with 1 μM PTX. (C) Continuation of red trace from Fig. 10, after activation of I_{PTX} in Na⁺ solution and 1.5 min of PTX washout in NMG⁺ solution; current did not decay for ~20 min in Na⁺ solution until 1 mM ouabain was added.
pump current in HEK293 cells (Kocksämper et al., 1997) made accurate measurements difficult, we used guinea-pig ventricular myocytes instead (e.g., Gadsby and Nakao, 1989), and exploited the rapid reversibility of dihydroouabain (e.g., Gao et al., 1995). During strong activation of outward Na/K pump current under whole-cell current recording with 50 mM pipette Na⁺ and 5 mM MgATP, and 30 mM extracellular Cs⁺ (as K⁺ co-ender), exposure to 1 mM dihydroouabain caused a sudden inward current shift (Fig. 12 A) due to inhibition of the entire population of myocyte Na/K pumps. Dissociation of dihydroouabain after its washout then slowly restored outward pump current (t_{0.5} = 53 ± 7 s, n = 14, Fig. 12 D). Reapplication of dihydroouabain again abolished Na/K pump current and, once inhibition was complete, a patch of membrane was excised in the outside-out configuration with all pumps still inhibited. Upon exposure to 1 μM PTX, concomitant with removal of dihydroouabain (Fig. 12 B), the PTX-induced conductance was activated in the subpopulation of pumps in the patch more rapidly (t_{0.5} = 18 ± 2 s, n = 8) than the pump current had recovered in that myocyte’s entire pump population after washout of dihydroouabain alone. In agreement with the results in HEK293 cells (Fig. 10), PTX-induced conductance activation in myocyte patches was much faster in the absence of cardionic steroid (t_{0.5} = 2.5 s, Fig. 12, C and D; but with otherwise identical solutions) than after the preincubation with dihydroouabain (Fig. 12 B and D), confirming that the latter slower time course reflects dissociation of bound steroid (in the presence of PTX). The even slower time course of pump current reactivation after washout of dihydroouabain alone (Fig. 12, A and D) demonstrates that PTX speeds dihydroouabain dissociation from the pump–steroid complex (as similarly concluded above for strophanthidin; Fig. 11, A and B), and hence that the two ligands must be able to interact with the same Na/K pump at the same time.

**DISCUSSION**

To better understand PTX interactions with the Na/K pump, we used excised outside-out patches for optimal control of conditions and we systematically varied the nucleotides in the pipette and the ions at the two membrane surfaces. We found that the apparent affinity for PTX could be reproducibly varied over several orders of magnitude, and was highest when Na/K pumps were exposed to K⁺-free, Na⁺-containing external and internal solutions and to high cytoplasmic [MgATP]. We found that PTX and a cardionic steroid can simultaneously occupy the same Na/K pump, but that each weakens the binding of the other. We found that the narrowest part of the permeation pathway through the PTX-induced channel must be ~7.5 Å wide. We next discuss the implications of these results.

**Influence of Nucleotides on PTX–Na/K Pump Interactions**

MgATP is known to influence Na/K pump function both in the submicromolar range, at which concentrations it phosphorylates the pump in the presence of cytoplasmic-side Na⁺ (Post et al., 1965), as well as in the submillimolar range over which it promotes deoclusion and release of K⁺ toward the cytoplasmic side (Forbush, 1987a). Only the latter low-affinity action can be mimicked by poorly hydrolyzable nucleotides like AMP-PNP or ADP (Simons, 1975; Kaplan and Kenney, 1982). We have previously shown that application of millimolar MgATP, MgAMPPNP, or MgADP instantaneously increased (~6-fold) the open probability of PTX-bound pump channels in excised inside out patches with saturating [PTX] in the pipette (Artigas and Gadsby, 2003a), indicating that low-affinity nucleotide interaction persists in PTX-modified Na/K pumps. However,
several of our findings indicate that high-affinity MgATP action, hence at the phosphorylation site, can also influence the PTX–Na/K pump complex. Thus, despite the above-mentioned similar actions of MgATP, MgAMPPNP, and MgADP on the $P_o$ of PTX-induced channels, $K_{0.5PTX}$, a measure of the apparent affinity for PTX action, was at least 10-fold smaller in patches equilibrated with Na solutions containing 5 mM MgATP than in patches equilibrated ($\approx$10 min) with pipette solutions containing 2 mM MgAMPPNP or 2 mM MgADP (Fig. 8 C), suggesting that prior phosphorylation of Na/K pumps (expected with MgATP, but not MgAMPPNP or MgADP) influences their interaction with PTX. This conclusion is further supported by the finding that equilibration with pipette solutions containing different [MgATP] influenced $K_{0.5PTX}$ over both submicromolar and submillimolar concentration ranges, whereas varying [MgAMPPNP] affected $K_{0.5PTX}$ only within the submillimolar concentration range (Fig. 9). Moreover, because we found that the decay of PTX-induced current upon PTX washout (in K-containing external solution), which reflects PTX unbinding (Artigas and Gadsby, 2003a), occurred rapidly when the pipette solution contained either no nucleotides or 2 mM MgAMPPNP, but was severalfold slower when pipettes contained 1 $\mu$M or 5 mM MgATP (Artigas and Gadsby, 2003b), we conclude not only that phosphorylation of Na/K pumps strengthens their interaction with PTX, but also that PTX-bound Na/K pumps exposed to MgATP likely remain phosphorylated for minutes.

To further interpret the large variations in $K_{0.5PTX}$ we observed with different pipette solutions, we consider a simplified scheme (Scheme I) that treats the Na/K pump, once it has bound PTX, as a channel that is either closed (C) or open (O), ignoring the gating complexities evident in preliminary single-channel dwell-time histograms that likely arise from the presence of two separate channel gates (Artigas and Gadsby, 2003a). As no channel activity is seen in the absence of PTX, toxin must first bind to the pump enzyme (E) before it can elicit the open-channel conformation; for simplicity, we assume that toxin also unbinds from the closed-channel state; this assumption is consistent with the observations that PTX unbinding rates (e.g., Figs. 4 and 5) are much lower than opening and closing rates of PTX-bound channels (Fig. 3; cf. Artigas and Gadsby, 2003a). This simplest scheme thus comprises three states:

\[
\text{PTX} \overset{K_{dPTX}}{\longrightarrow} \overset{K_o}{\longrightarrow} \overset{K_{off}}{\longrightarrow} \text{E}
\]

**Scheme I**

At steady state, distributions among these conformations are determined both by the equilibrium dissociation constant, $K_{dPTX}$ (= $k_{off}/k_{on}$, the ratio of dissociation and association rate constants), for PTX binding to state E, and by the equilibrium constant, $K_o$, between closed- and open-channel states of the PTX-bound pump, $K_o$ (= $k_o/k_{oc}$, the ratio of the rate constants for channel opening and channel closing). Accordingly, the apparent dissociation constant for palytoxin ($K_{0.5PTX}$) will depend both on the open probability of a PTX-bound pump channel ($P_o = k_o/(k_o + k_c) = K_o/(K_o + 1)$) and on $K_{dPTX}$, and will approach the latter only if $P_o$ is very small (i.e., if $K_o$ is very small):

\[
K_{0.5PTX} = K_{dPTX}/(1 + K_o) = (1 - P_o)K_{dPTX}. \quad (1)
\]

This relation allows estimation of $K_{dPTX}$ from the $K_{0.5PTX}$ values determined in Figs. 8 C and 9 under conditions for which $P_o$ is also known. From single-channel current recordings in outside-out patches from cardiac myocytes, with Na-containing solutions, $P_o$ was $\sim0.9$ ($K_o$ $\sim9$) for PTX-induced channels in the presence of 5 mM MgATP, but was reduced to $P_o \simeq 0.2$ in the absence of nucleotide (Artigas and Gadsby, 2003a). The rapid sixfold increase in PTX-induced current in larger inside-out patches upon suddenly switching from ATP-free to 5-mM MgATP-containing solution (Artigas and Gadsby, 2003a) suggests that a reasonable estimate for $P_o$ is $\sim0.15$ ($\simeq 0.9/6$; i.e., $K_o \sim 0.18$) in the absence of nucleotide. Thus, for 5 mM MgATP, $K_{0.5PTX} \sim 30$ pM = 0.1$K_{dPTX}$ yielding $K_{dPTX} \sim 300$ pM. In the absence of nucleotide, $K_{0.5PTX}$ averaged $\sim 19$ nM = 0.85$K_{dPTX}$, which yields $K_{dPTX} \sim 22$ nM. Because addition of 1 $\mu$M MgATP only slightly increased PTX-induced current in inside-out patches (by $<10\%$ of the maximal increase seen with 5 mM MgATP; Artigas and Gadsby, 2003a), the channel $P_o$ in 1 $\mu$M MgATP may be assumed comparable to that in the absence of nucleotide, and so the $K_{0.5PTX}$ of $\sim 250$ pM in 1 $\mu$M MgATP (Fig. 9) corresponds to a $K_{dPTX} \sim 300$ pM. Like MgATP, 2 mM MgAMPPNP increased macroscopic PTX-induced current $\sim 6$-fold in inside-out myocyte patches (Artigas and Gadsby, 2003a), indicating that $P_o$, in high [MgAMPPNP] is close to that high [MgATP], and so the mean $K_{0.5PTX}$ value of $\sim 760$ pM (Fig. 8) implies that $K_{dPTX}$ is $\sim 8$ nM in MgAMPPNP.

These values allow parsing of the influences on $K_{dPTX}$ of pump phosphorylation and of low-affinity nucleotide binding; if we make the simplifying assumptions that 1 $\mu$M MgATP supports phosphorylation but negligible occupancy of the low-affinity sites, that 5 mM MgATP supports both phosphorylation and low-affinity binding, and that 2 mM MgAMPPNP supports low-affinity binding but no phosphorylation. Within these assumptions, phosphorylation of the pump alone appears responsible for the $\sim 70$-fold smaller $K_{dPTX}$ seen with 1 $\mu$M MgATP ($\sim 300$ pM) than with no nucleotides ($\sim 22$ nM); this stimulatory influence of phosphorylation (at the cytoplasmic side of the pump) on
PTX binding (at the extracellular side) seemed little altered by occupancy of low-affinity nucleotide sites (at the cytoplasmic side), viz. \( K_{\text{PTX}}^{\text{E}} \) of \(~300\) pM with 5 mM MgATP vs. \(~8\) nM with 2 mM MgAMPPNP, a \(~30\)-fold enhancement of PTX affinity. In contrast, the affinity for PTX seemed only modestly enhanced by low-affinity nucleotide binding, regardless of whether the pumps were phosphorylated (\( K_{\text{PTX}}^{\text{E}} \sim300\) pM with either 1 \( \mu \)M or 5 mM MgATP) or unphosphorylated (\( K_{\text{PTX}}^{\text{E}} \sim22\) nM without nucleotides vs. \(~8\) nM with 2 mM MgAMPPNP). Similarly, in work on red blood cell membranes, ATP, and to a lesser extent ADP, was shown to promote PTX-mediated cation flux (Chhatwal et al., 1983), to enhance \(^{125}\text{I}-\text{PTX} \) binding (Böttinger et al., 1986), and to promote opening of channels in inside-out patches when PTX was in the pipette (Kim et al., 1995).

As already mentioned, this enhanced affinity of PTX for phosphorylated pumps seems at least partly attributable to slowed dissociation. This is evident whether PTX unbinding is monitored simply from current decay upon PTX withdrawal in external \( \text{Na}^+ \) (\(~10\)-fold slower with 5 mM MgATP in the pipette than with 2 mM MgAMPPNP or no nucleotides; e.g., Figs. 4 and 8, above) or assessed by residual current in external \( \text{Na}^+ \) after brief exposures to external \( \text{K}^+ \) (severalfold slower with 1 \( \mu \)M or 5 mM MgATP than with 2 mM MgAMPPNP or no nucleotides; Artigas and Gadsby, 2003b).

**PTX Alters the Na/K Pump’s Phosphorylation Status**

Further implications of the large influence on PTX affinity of phosphorylation by 1 \( \mu \)M MgATP become clear when states corresponding to those in Scheme I are linked by explicit phosphorylation reactions (justified by the above conclusion that PTX-bound pumps appear to remain phosphorylated). In Scheme II, the subscript \( \text{P} \) identifies phosphorylated states (\( \text{EP}, \text{CP}, \text{OP} \)), and the \( K_{\text{pho}} \) are lumped equilibrium constants that incorporate all steps involved in pump phosphorylation and dephosphorylation.

Because the \( P_e \) of PTX-bound pump channels is comparable with 0 or 1 \( \mu \)M MgATP (hence \( K_{\text{pho}} \); Artigas and Gadsby, 2003a), microscopic reversibility dictates that the equilibrium constants that describe phosphorylation of open and closed channel states, \( K_{\text{pho}}^{\text{E}} \) and \( K_{\text{pho}}^{\text{C}} \), must also be similar. By the same token, the very different \( K_{\text{PTX}}^{\text{E}} \) values of phosphorylated and unphosphorylated pumps (\( K_{\text{PTX}}^{\text{E}} \sim300\) pM vs. \( K_{\text{PTX}}^{\text{E}} \sim22\) nM; see above) means that the equilibrium constant for phosphorylation of unmodified pumps (\( K_{\text{pho,E}}^{\text{K}} \)) must differ strongly from those of toxin-bound pumps (\( K_{\text{pho,C}}^{\text{K}} \), and hence also \( K_{\text{pho,E}}^{\text{K}} \)), via:

\[
K_{\text{PTX}}^{\text{E,K}} \cdot K_{\text{pho,E}}^{\text{K}} = K_{\text{PTX}}^{\text{E,K}} \cdot K_{\text{pho,C}}^{\text{K}}.
\]

Our numbers yield \( K_{\text{pho,C}}^{\text{K}} = 70K_{\text{pho,E}}^{\text{K}} \), which indicates a substantial shift toward the phosphorylated state in the PTX–pump complex. The lumped phosphorylation–dephosphorylation reaction comprises \( \text{Na}^+ \)- and ATP-binding steps, phosphoryl transfer, and dephosphorylation, all steps that have been well characterized in unmodified pumps, though not after modification by PTX (but see Tosteson et al., 2003). In either case, the high (150 mM) pipette [\( \text{Na}^+ \]), and the presence of ATP but nominal absence of ADP ought to render the phosphorylation steps kinetically irreversible. A reasonable explanation, then, for the \(~70\)-fold enhanced phosphorylation constant of PTX-bound pumps, is that dephosphorylation of the pump is somehow impaired once PTX is bound. The presence of 160 mM external \( \text{Na}^+ \) is likely to drive dephosphorylation of the normal, unmodified pumps via a \( \text{Na},\text{Na}-\text{ATPase} \) cycle, in which occlusion of 2 \( \text{Na}^+ \) (in place of the normal 2 \( \text{K}^+ \)) is associated with loss of phosphate. If dephosphorylation similarly requires ion occlusion in PTX-bound pumps, then the apparent propensity of PTX to interfere with closure of the external gate to the cation-binding cavity (Artigas and Gadsby, 2003a,b) offers a possible mechanism for how PTX may impair both occlusion and dephosphorylation. It is not clear how this inference of enhanced pump phosphorylation by MgATP in the presence of PTX relates to a recent conclusion that PTX impaired phosphorylation of purified Na,K-ATPase from MgATP, but enhanced phosphorylation from P (Tosteson et al., 2003).

**Nucleotide Interactions with their Low-affinity Binding Site on the Na/K Pump**

Analysis of low-affinity nucleotide interactions requires that states appropriate for 1 \( \mu \)M MgATP and those expected at saturating (i.e., 5 mM) MgATP be linked by nucleotide binding reactions (Scheme III); an analogous scheme can be used for a comparison of observations without nucleotide and with 2 mM MgAMPPNP.
As already noted, $K_{\text{PTX}}$ for phosphorylated pumps ($K_{\text{PTX-P}}$ in Scheme III) is $\sim$300 pM at 1 $\mu$M MgATP and was apparently unaffected by low-affinity ATP binding, being also $\sim$300 pM at 5 mM MgATP ($K_{\text{PTX-ATP}}$ in Scheme III). In unphosphorylated pumps, too, $K_{\text{PTX}}$ was only little affected by low-affinity nucleotide binding, being $\sim$22 nM without nucleotides and $\sim$8 nM with 2 mM MgAMPPNP. For both these cases, then, microscopic reversibility (applied to Scheme III) requires that the equilibrium dissociation constants for low-affinity nucleotide binding to the unmodified pump, $K_{\text{LE}}$, and to the closed state of the PTX-bound pump channel, $K_{\text{LC}}$, be roughly the same. However, channel open probability (and hence $K_o$) is greatly increased upon nucleotide binding to the low affinity site (Artigas and Gadsby, 2003a), yielding values of $K_o\approx 0.18$, and $K_{oP,ATP}\approx 9$. Because microscopic reversibility forces:

$$K_{\text{LC}}K_{oP} = K_{\text{LO}}K_{oP,ATP},$$

we conclude that $K_{\text{LC}}\approx 50 K_{\text{LO}}$, where $K_{\text{LO}}$ is the dissociation constant for low-affinity binding of MgATP to the open-channel state. In other words, MgATP binds with 50-fold higher affinity to the open state of the PTX-bound pump channel than to its closed state (or to the unmodified pump). But can we estimate absolute values?

By considering only the PTX-bound states in Scheme III, we can express the open probability, i.e., the steady-state fractional occupancy of states $O_p$ and $O_{P,ATP}$ as a function of [ATP]:

$$P_o = ([\text{ATP}] + K_{\text{LO}})/(K_{\text{LO}}(1 + K_{oP})/K_{oP}) + ([\text{ATP}](1 + K_{oP,ATP})/K_{oP,ATP}).$$

If occupancy of state $O_{P,ATP}$ is assumed negligible at 1 $\mu$M ATP, and of state $O_p$ is negligible at 5 mM ATP, this can be written as

$$P_o = ([\text{ATP}] + K_{\text{LO}})/((K_{\text{LO}}/P_{\text{omin}}) + ([\text{ATP}]/P_{\text{omax}}),$$

where $P_{\text{omin}}$ is the open probability at 0 (or 1 $\mu$M) ATP, and $P_{\text{omax}}$ is the $P_o$ at 5 mM ATP. Because PTX dissociates so slowly, the variation of PTX-induced macroscopic current (reflecting the change in $P_o$, $\Delta P_o$) with changes of [ATP] bathing inside-out patches, could be approximated by the Michaelis function:

$$\Delta P_o = (P_{\text{omax}} - P_{\text{omin}})[\text{ATP}]/(K_{\text{ATP}} + [\text{ATP}]),$$

where $K_{\text{ATP}} = K_{\text{LO}}P_{\text{omax}}/P_{\text{omin}}$.

The fit gave $\sim$20 $\mu$M for $K_{\text{ATP}}$ (Artigas and Gadsby, 2003a), and since $P_{\text{omax}}/P_{\text{omin}}\approx 6$, the dissociation constant for low-affinity ATP binding to the open-channel state, $K_{\text{LO}}$, is $\sim$3 $\mu$M, which in turn means that $K_{\text{LE}}$, for the closed state, is $\sim$150 $\mu$M. Because the latter should also apply to the unmodified pump (as $K_{\text{LC}}\approx K_{\text{LE}}$ above), it is of interest that comparable values have been obtained for the apparent affinity with which ATP accelerates Na,K-ATPase activity (Post et al., 1972; Mo- czyóldowskí and Fortes, 1981) or ouabain-sensitive K/K exchange (Simons, 1974), or accelerates deocclusion of K$^+$ toward the pump’s cytoplasmic surface (Forbush, 1987a,b), or activates pump current amplitude (Friedrich et al., 1996). Insofar as the rate-limiting step in the entire Na,K-ATPase cycle is K$^+$ deocclusion, ATP may be expected to exert its effect in all these instances by binding at the low-affinity site. Correspondingly, we interpret the ATP-induced increment in the $P_o$ of PTX-induced channels as reflecting opening of the cytoplasmic-side gate in response to low-affinity binding of the nucleotide to the PTX-pump complex.

Because cytoplasmic K$^+$ deocclusion normally follows dephosphorylation in unmodified Na/K pumps, it is not surprising that similar considerations apply to AMPPNP (which cannot support phosphorylation). Thus, we found that 2 mM MgAMPPNP essentially mimics the effects of low-affinity ATP binding in that it little altered $K_{\text{PTX}}$ (see above) but markedly increased, $\sim$6-fold, the $P_o$ of PTX-bound pump channels (Artigas and Gadsby, 2003a).

Influence of External K$^+$ or Cardiotonic Steroids on PTX–Na/K Pump Interactions

A comparably detailed analysis of the reductions in apparent PTX affinity we observed (all in the presence of 5-mM pipette MgATP) on replacing external and/or internal Na$^+$ by K$^+$, or upon exposure to cardiotonic steroids, is presently not possible because we know little about the $P_o$ of PTX-bound pump channels under those conditions. External K$^+$ ions are able to shut PTX-bound pump channels, and hence lower $P_o$, in the absence of nucleotides (Artigas and Gadsby, 2003a), and also in the presence of MgAMPPNP (Artigas and Gadsby, 2003b), but we lack evidence for K$^+$-induced changes in $P_o$ in the presence of high [MgATP]. Nevertheless, because 5 mM MgATP was always present in the conditions under consideration, if we use all-Na$^+$ solutions as our reference point ($P_o\approx 0.9$), Eq. 1 indicates that even a reduction in $P_o$ to zero could account for no more than a 10-fold increase in $K_{\text{ATP}}$. The observed $\sim$100-fold increase in $K_{\text{ATP}}$ on replacing external Na$^+$ by K$^+$, regardless of whether the principal internal cation was Na$^+$ ($\sim$30 pM increased to $\sim$3 nM; Figs. 4 C and 6 D) or K$^+$ ($\sim$500 pM increased to $\sim$30 nM; Figs. 6 B and 5 C), therefore imply that external K$^+$ ions act on the Na/K pump to reduce its true affinity for PTX. This interpretation is supported by the parallel finding that external K$^+$ also acted to accelerate ($\sim$30-fold, with either internal Na$^+$ or K$^+$; e.g., Figs. 4 A
and 5 A) the slow deactivation of PTX-induced current after PTX washout that likely reflects PTX unbinding. These results confirm earlier demonstrations of antagonism by external K⁺ of PTX action (Ahnert-Hilger et al., 1982) or of ¹²⁵I-PTX binding (Böttinger et al., 1986), and of relief of PTX-mediated inhibition of ATPase activity by increasing [K⁺] (Ishida et al., 1983).

We have not measured the Pₐ of PTX-bound pump channels in the presence of cardiotonic steroids. But, our finding that higher [PTX] speeds the (still slowed) opening of channels in pumps initially complexed with a cardiotonic steroid (Fig. 11, A and B), apparently by speeding dissociation of that complex, demonstrates not only that PTX and steroid can occupy the same Na/K pump molecule (as does the steroid-induced speeding of PTX dissociation; Fig. 11 C), but also that such a PTX-bound pump channel does not open until the steroid has unbound. In other words, the bound steroid must sustain a closed conformation of the PTX-bound pump channel, which in turn implies that binding of the steroid would lower Pₐ of pump channels opened by PTX. Though that fall in Pₐ would contribute to an increase in Kₒ,PTX (though <10-fold), the marked steroid-mediated reduction we observed in apparent affinity for PTX action (e.g., Figs. 10 and 11, as previously reported: Chhatwal et al., 1983; Habermann, 1989) and the acceleration of PTX unbinding (Fig. 11 C) both indicate that, when also bound to the Na/K pump, the steroids directly increase Kₒ,PTX.

In unmodified Na/K pumps, replacement of external Na⁺ by K⁺ promotes K⁺-ion occlusion in the dephosphorylated conformation E₂(K⁺) (e.g., Beaugé and Glynn, 1979), whereas, in all-Na⁺ solutions with MgATP, addition of cardiotonic steroids promotes Na⁺-ion occlusion in the phosphorylated conformation EₚP(Na⁺) (e.g., Stürmer and Apell, 1992). The salient common factor is closure of an external gate, which can also explain the common tendencies of these two maneuvers both to lower the Pₐ of PTX-bound pump channels and to destabilize the PTX–Na/K pump complex.

**Which Na/K Pump Conformation Binds PTX with Highest Affinity?**

Previous authors have favored E₁ (e.g., Scheiner-Bobis and Schneider, 1997) or E₂ (e.g., Wang and Horisberger, 1997) or both (Habermann, 1989) conformations of the Na/K pump as the state(s) to which PTX prefers to bind (for review see Tosteson, 2000). Given that an unmodified Na/K pump can support a maximal uphill Na⁺-ion efflux of ≈5 × 10⁸ ions s⁻¹, whereas the channel in a PTX-bound Na/K pump can pass a dissipative Na⁺-ion influx of ≈0.8 pA (Fig. 3 C; i.e., ≈5 × 10⁶ ions s⁻¹), the probability that such an open-channel conformation can exist in an unmodified pump must be extremely small, <10⁻⁴. In other words, the Na/K pump conformation stabilized by PTX is not likely to be any of the states visited in the familiar Post-Albers kinetic scheme for ion transport by the Na/K pump. Indeed, our results show that PTX binds with highest affinity to phosphorylated Na/K pumps exposed to K⁺-free, high [Na⁺] external and internal solutions with MgATP. In these conditions, the pumps may be expected to largely populate EₚP conformations in the steady state. As mentioned, the subsequent slow current decline on PTX withdrawal, reflecting slow dissociation of PTX, implies that the pumps remained phosphorylated as long as PTX was bound, and hence that the ATPase cycle was stalled before the dephosphorylation step. But high concentrations of PTX could also open pump channels during exposure to Na⁺-free, high [K⁺] internal and external solutions, in which, despite the presence of 5 mM internal MgATP, the predominant steady-state conformations are expected to be nonphosphorylated E₃ states (equally true for all Na⁺ solutions with no, or nonhydrolyzable, nucleotide) and ATPase cycling should also be precluded. In terms of the alternating-gate scheme of pump-mediated Na/K transport, in which access to binding sites is controlled by an extracellular-side gate in E₂ conformations and by a cytoplasmic-side gate in E₁ conformations, PTX-induced channel states with both gates open must be hybrids with features of both E₁ and E₂ conformations. Despite the absence of ATPase cycles (Ishida et al., 1983), however, PTX-bound pumps are not restricted to a single conformational state, as ligand-mediated gating events persist that appear to resemble normal partial reactions of the pump associated with occlusion and deocclusion of transported cations (Artigas and Gadsby, 2003a). Because PTX-bound pump channels in physiologically relevant solutions, with high [Na⁺] and with millimolar [MgATP], display a Pₐ of ~0.9, it appears that PTX has evolved to bind most tightly to, and hence to most effectively stabilize, the conformation that is most lethal to animal cells.

**Simultaneous Na/K Pump Occupancy by a Cardiotonic Steroid and PTX**

While earlier work pointed to some form of competition between PTX and cardiotonic steroids (for review see Habermann, 1989; Tosteson, 2000), the results presented here establish that both of these large compounds can simultaneously interact with the same Na/K pump. The clearest demonstration is that each can accelerate the unbinding from the Na/K pump of the other. Because both PTX (Muramatsu et al., 1984) and cardiotonic steroids (e.g., Forbush et al., 1978) are believed to exert their action by binding to the external surface of the pump, we must conclude that a single
Na/K pump can simultaneously accommodate at least some portion of a \( \sim 2.7 \text{kD} \) PTX molecule and some part of a \( \sim 0.5 \text{kD} \) cardiotonic steroid molecule. Although a combination of mutagenesis (e.g., Price and Lingrel, 1988; Canessa et al., 1993; Palasis et al., 1996; Koenderink et al., 2000) and structure-function (e.g., Middleton et al., 2000; Sweadner and Donnet, 2001; Ball et al., 2003) analyses are helping to identify the docking sites for cardiotonic steroids, there is as yet no information about where or how the large, extended-chain PTX molecule interacts with the exterior of the Na/K pump.

**Implications of Permeation Data**

The single-channel conductance of \( \sim 7 \text{ pS} \) found here for PTX-induced channels in native Na/K pumps of guinea-pig ventricular myocytes during exposure to all-Na\(^+\) solutions fits with the 7–10 pS values reported previously for various preparations (e.g., Ikeda et al., 1988; Muramatsu et al., 1988; Rettinger and Schwarz, 1994; Kim et al., 1995; Hirsh and Wu, 1997; Wang and Horisberger, 1997). Use of the outside-out patch configuration in the present work allowed unambiguous identification of PTX-induced pump channel currents as those elicited exclusively upon addition of the toxin. A novel finding was the unequivocal demonstration that N-methyl-d-glucamine, with an average diameter of \( \sim 7.3 \text{ Å} \), could pass through the open channel, whereas the \( \approx 8-\text{Å} \) diameter tetraprolyl ammonium could not. This prompts further questions. First, if the pore is so wide, why is the single-channel conductance for Na\(^+\) only \( \sim 7 \text{ pS} \)? A possible explanation is that more than one ion can occupy the pore at a time, and that ions interact within the pore. Measurements of tracer flux ratio exponents should help evaluate this possibility (Rakowski et al., 2003).

Second, is this wide pore part of the normal ion pathway negotiated by the transported Na\(^+\) and/or K\(^+\) ions? If it is, then the observed throughput rate of \( \approx 5 \times 10^6 \text{ Na}\(^+\) ions s\(^{-1}\) provides a lower estimate for the dissociation rate of the tightest Na\(^+\)-binding sites within the pore; assuming a diffusion-limited binding rate of 10\(^6\) M\(^{-1}\) s\(^{-1}\) at those sites, their dissociation constant could be no smaller than 5 mM. As K\(^+\) ions are conducted even more rapidly than Na\(^+\), they must bind even more loosely within the pore. These considerations indicate that, if the pore is part of the normal ion translocation pathway, the Na\(^+\)- and/or K\(^+\)-ion binding sites with submillimolar apparent affinity in the unmodified Na/K pump (e.g., Bühler and Apell, 1995; Schneeberger and Apell, 2001) must be characteristic of conformations in which one or other of the pump’s two gates is closed. Initial evidence that the pore of PTX-induced channels comprises at least some segment of the ion-translocation pathway comes from cysteine-scanning mutagenesis studies (Guennoun and Horisberger, 2000; 2002). PTX was found to render accessible to small water-soluble sulfhydryl-specific reagents cysteine residues introduced at positions in the fifth and sixth transmembrane helices that are believed to help coordinate the transported ions, on the basis of site-specific mutagenesis experiments (e.g., Nielsen et al., 1998; Pedersen et al., 1998) and of homology modelling (Ogawa and Toyoshima, 2002; Rakowski and Sagar, 2003) using the crystal structures of the related SERCA pump (Toyoshima et al., 2000; Toyoshima and Nomura, 2002). Additional evidence that the pore of PTX-bound pump channels usurps the normal ion translocation path is provided by our demonstration that the \( P_\text{o} \), of those channels can be modulated by ligands of the Na/K pump in a manner consistent with their known action to favor occlusion or deoclusion of the transported ions (Artigas and Gadsby, 2003a,b). Interestingly, the SERCA pump structures appear to incorporate relatively wide access channels. Thus, a cavity was observed in the 8-Å resolution E2-decavanadate SERCA pump structure (Zhang et al., 1998), consistent with a water-filled access channel to the ion binding sites from the lumen (equivalent to the extracellular side in the Na/K pump). Also, the high-resolution E2-thapsigargin structure (Toyoshima and Nomura, 2002) contained a water-accessible channel, lined with negatively charged residues, leading toward the binding sites from the cytoplasmic side. These access channel-like structures in SERCA pumps are in accord with our data that suggest the presence of a \( \sim 7.5-\text{Å} \) wide ion pathway in a PTX-modified Na/K pump. If the PTX-induced pore does indeed comprise at least some part of the binding pocket(s) occupied by occluded Na\(^+\) and/or K\(^+\) ions during their transport by the unmodified pump, then the improved understanding of PTX’s interaction with the Na/K pump afforded by the present work should now allow use of PTX to help reveal the locations, structures, and mechanisms of the Na/K pump’s gates that alternately occlude and deoclude the transported ions.

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