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Palytoxin acts on Na$^+$,K$^+$-ATPase but not non-gastric H$^+$,K$^+$-ATPase

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Abstract

Palytoxin (PTX) opens a pathway for ions to pass through Na,K-ATPase. We investigate here whether PTX also acts on non-gastric H,K-ATPases. The following combinations of cRNA were expressed in *Xenopus laevis* oocytes: *Bufo marinus* bladder H,K-ATPase α_2- and Na,K-ATPase β_2-subunits; *Bufo* Na,K-ATPase α_1- and Na,K-ATPase β_2-subunits; and *Bufo* Na,K-ATPase β_2-subunit alone. The response to PTX was measured after blocking endogenous *Xenopus* Na,K-ATPase with 10 μM ouabain. Functional expression was confirmed by measuring ^{86}Rb uptake. PTX (5 nM) produced a large increase of membrane conductance in oocytes expressing *Bufo* Na,K-ATPase, but no significant increase occurred in oocytes expressing *Bufo* H,K-ATPase or in those injected with *Bufo* β_2-subunit alone. Expression of the following combinations of cDNA was investigated in HeLa cells: rat colonic H,K-ATPase α_1-subunit and Na,K-ATPase β_1-subunit; rat Na,K-ATPase α_2-subunit and Na,K-ATPase β_2-subunit; and rat Na,K-ATPase β_1 or Na,K-ATPase β_2 subunit alone. Measurement of increases in ^{86}Rb uptake confirmed that both rat Na,K- and H,K-pumps were functional in HeLa cells expressing rat colonic HKα_1/NKβ_1 and NKα_2/NKβ_2. Whole-cell patch clamp measurements in HeLa cells expressing rat colonic HKα_1/NKβ_1 exposed to 100 nM PTX showed no significant increase of membrane current and there was no membrane conductance increase in HeLa cells transfected with rat NKβ_1 or rat NKβ_2 subunits alone. However, in HeLa Cells expressing rat NKα_2 NKβ_2, outward current was observed after pump activation by 20 mM K^+ and a large membrane conductance increase occurred after 100 nM PTX. We conclude that non-gastric H,K-ATPases are not sensitive to palytoxin when expressed in these cells whereas palytoxin does act on Na,K-ATPase.

Introduction

The PII$_C$-type ion-motive ATPase subgroup (also termed X,K-ATPases) includes the ubiquitous Na,K-ATPase, the gastric H,K-ATPase found in parietal cells, and the non-gastric H,K-ATPases expressed in distal colon (Crowson & Shull 1992). Non-gastric H,K-ATPase is over-expressed under pathophysiological conditions such as chronic hypokalemia, NaCl deficiency or renal acidosis (Silver and Soleimani 1999). Several variants of non-gastric H,K-ATPases have been identified and isolated from the amphibian toad bladder, human skin, guinea pig and rabbit distal colon. X,K-ATPases require another polypeptide, the glycosylated β-subunit to be expressed and functional within the plasma membrane (Geering, 1998) where they maintain K^+ homeostasis and intracellular ionic composition. The closely related PII$_A$-type ATPase subgroup, including the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA), has characteristics similar to those of PII$_C$-type ATPases. The structure of their catalytic α-

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subunits shows a high level of sequence similarity (Sweadner and Donnet 2001). They exchange Ca^{2+} or Na^+ in exchange for H^+ or K^+ by using the energy derived from ATP hydrolysis. PII_C- and PII_A-type ATPases are inhibited by vanadate acting at the ATP binding site (Cantley, et al. 1978). Experimental data have shown that both rat colonic and *Bufo* bladder H,K-ATPases can transport Na^+ rather than H^+ ions (Cougnon, et al. 1998; Spicer, et al. 2001). Despite these similarities, however, Na,K-ATPase differs in several important aspects from both gastric and non-gastric H,K-ATPases. The Na,K-ATPase functions with a transport stoichiometry of $3Na^+/2K^+$ resulting in outward transport of one net charge per pump cycle (Post and Jolly 1957; Rakowski, et al. 1989), whereas H,K-ATPases have a $1K^+/1H^+$ or $2K^+/2H^+$ stoichiometry resulting in net electroneutral exchange (Sachs, et al, 1976; Rabon, et al. 1982; Burnay et al. 2001). In polarized epithelial tissues, Na,K-ATPase is mainly located in the basolateral plasma membrane (Gottardi and Caplan 1993), whereas H,K-ATPases are present primarily at the apical surfaces (Caplan, 1997; Smolka, et al. 1983; Pestov et al. 2002). *Bufo*, rat, and human ngH,K-ATPases are moderately sensitive to ouabain, and both *Bufo* and rat ngH,K-ATPases can be inhibited by high concentrations of SCH-28080 (Del Castillo, et al. 1991; Codina, et al. 1996).

At picomolar concentrations the highly potent marine toxin, palytoxin (PTX) binds to the Na,K-ATPase and converts it from an ion pump into an ion channel (Habermann 1989; Wang and Horisberger 1997; Artigas and Gadsby 2003). This greatly increases the membrane conductance and results in a net inward current carried by Na^+. The gating of palytoxin-induced ion channels can be modulated by Na,K-ATPase ligands such as Na^+ or ATP (Artigas and Gadsby 2003, 2004; Hilgemann, 2003). Palytoxin-induced membrane conductance can also be inhibited by ouabain or K^+ ions (Ozaki, et al., 1985; Artigas and Gadsby, 2003). Experimental data from cysteine-scanning accessibility studies combined with structural modeling based on the 3-D structure of sarco-endoplasmic reticulum Ca^{2+}-ATPase, SERCA (Guennoun and Horisberger 2000, 2002) have suggested that the PTX-induced ion channel includes at least a part of the Na^+ and K^+ transport pathway. Reports on the effect of palytoxin on the apical versus basolateral side of polarized epithelial cells from kidney (LLC-PK1), provide additional evidence that the site of palytoxin action is the Na,K-ATPase (Mullin, et al 1991). However, it has been reported that palytoxin acts on both distal and proximal parts of the descending colon (Scheiner-Bobis, et al., 2002). Extensive pharmacological characterizations of colonic and other H,K-ATPases found in collecting duct have yielded conflicting conclusions regarding its sensitivity to ouabain (Codina J., et al., 1996; Cougnon, et al., 1996; Del Castillo, et al., 1991). Hence definitive conclusions cannot be drawn regarding the site of PTX action based on studies in colon and collecting duct and further investigation of PTX action on both Na,K-ATPase and H,K-ATPase in tissues with well-defined expression of these transport proteins is warranted.

The goal of the present study is to determine whether palytoxin increases the conductance of non-gastric H,K-ATPases or acts only on Na,K-ATPase. We expressed *Bufo* ngH,K-ATPase, Na,K-ATPase or Na,K-ATPase β_2-subunit alone in *Xenopus* oocytes, and in addition, we expressed rat colonic H,K-ATPase, Na,K-ATPase and Na,K-ATPase β_1 or β_2 subunits alone in HeLa cells. *Bufo* and rat Na,K-ATPases are both ouabain-resistant. Both *Xenopus* oocyte and HeLa Na,K-ATPase are ouabain-sensitive. Thus we can inhibit the endogenous sodium pump with a low dose (*e.g.* 10 μM) of ouabain without completely blocking the exogenous Na,K-ATPase in the cells used for these studies. To investigate the action of PTX we measured conductance changes using the two microelectrode voltage-clamp technique in *Xenopus* oocytes and whole cell current in HeLa cells using the patch clamp technique. We also examined models of Na,K- and ngH,K-ATPase based on the known 3-D structure of SERCA in order to determine if major difference exists between the two that could be related to their ability to respond to PTX.

Materials and Methods

Plasmid construction

Full-length *c*DNAs encoding rat Na,K-ATPase α_1-subunit (NKα_1), rat Na,K-ATPase β_1-subunit (NKβ_1), rat colonic H,K-ATPase α_2-subunit (HKα_2), and rat Na,K-ATPase β_2-subunit (NKβ_2) were digested by restriction enzymes that conserve the 5′-Kozak translation initiation sequence, the methionine start-codon, and the stop-codon-3′ ends of the genes of interest. Using T4 DNA ligase, we inserted each *c*DNA fragment into the previously linearized pcDNA3.1 (+). In order to achieve high expression levels in mammalian cells, we used a restriction enzyme that cut within multiple cloning sites conserving the CMV promoter and BGH polyadenylation signal. The coding regions of *Bufo* Na,K-ATPase α_1-subunit (NKα_1), Na,K-ATPase β_2-subunit (NKβ_2) or *Bufo* bladder H,K-ATPase α_2-subunit (HKα_2) *c*RNAs were inserted into pSD5 vector with SP6 promoter allowing high levels of protein expression in *Xenopus* oocytes.

Expression systems

Xenopus oocytes were microinjected with *Bufo* NKα_1/NKβ_2 *c*RNAs to over-express *Bufo* Na,K-ATPase, and with *Bufo* HKα_2/NKβ_2 *c*RNAs to over-express *Bufo* (bladder) ngH,K-ATPase, or *Bufo* β_2-subunit (NKβ_2) *c*RNA alone. HeLa cells were transiently transfected with rat NKβ_1-subunit *c*DNA and co-transfected with a total of 2 mg *c*DNA of rat NKα_1/NKβ_1 to over-express rat Na,K-ATPase, or with a total of 2 mg *c*DNA of HKα_2/HKβ_2 to over-express rat (colonic) ngH,K-ATPase, using PolyFect reagent (Qiagen) following the protocol described by the vendor.

^{86}Rb uptake measurements were performed to ensure that we achieved high levels of functional expression of both *Bufo* ngH,K- and Na,K-ATPase in *Xenopus* oocytes and rat ngH,K- and Na,K-ATPase in HeLa cells. *Xenopus* oocytes expressing *Bufo* bladder H,K-ATPase, Na,K-ATPase or β_2-subunit alone were loaded with Na^+ by 2-h incubation in a K^+-free/Ca^{2+}-free solution containing (in mM): 90 NaCl and 0.5 EGTA. Na^+-loaded oocytes were transferred to a solution containing (in mM): 5 KCl, 90 NaCl, 1 $CaCl_2$, 10 HEPES, pH 7.4, 0.2 μM ouabain (to inhibit endogenous Na,K-ATPase), and 10 μM bumetanide (to inhibit ^{86}Rb-uptake mediated by the Na-K-2Cl co-transporter). Oocytes were incubated 12 minutes with ^{86}Rb (5 μCi/ml) at room temperature, and washed with a solution containing (in mM): 90 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH 7.4. Individual oocytes were then dissolved in 0.5 % SDS and ^{86}Rb uptake was determined by scintillation counting. HeLa cells grown in 24-well cluster dishes at 60-80% confluency were transiently transfected as described above. Three days later, ^{86}Rb uptake measurements were performed using a wash tray according to Sangan, et al. (Sangan, et al., 2000). After drilling a hole in each cover-well (~12.5 mm), we inserted a plastic test tube (#2052, Falcon) and then glued it into position. This allowed us to fill each tube with wash-solutions and invert the whole assembly on the 24-well culture dish containing transfected HeLa cells. The solution obtained in each tube of the wash tray was transferred into individual test wells.

Steady-state Voltage Clamp measurements

Xenopus oocytes were microinjected with *Bufo* NKα_1/NKβ_2, HKα_2/NKβ_2, and NKβ_2 *c*RNAs encoding for *Bufo* Na,K-ATPase, bladder ngH,K-ATPase, and Na,K-ATPase β_2-subunit respectively. Three or four days later, the steady-state current activated by 10 mM extracellular K^+ was measured at holding potential of -50mV using the two-electrode voltage clamp technique. The experimental solution contained (in mM): 100 Na^+-gluconate, 0.82 $MgCl_2$, 0.41 $CaCl_2$, 10 NMDG/HEPES, 5 $BaCl_2$, 10 TEA-Cl_2, and 0.2 μM ouabain (to block endogenous *Xenopus* Na,K-pumps). Ba^{2+} and TEA^+ were present to block passive K^+ channels (Rakowski and Paxson, 1988) so that the current produced by ouabain resistant *Bufo* Na,K pumps could be measured upon addition of extracellular K^+. An aliquot of 100 μM PTX

(Sigma) was thawed just prior to each experiment and diluted to a final concentration of 5 nM in the external K^+-free solution containing 0.002% BSA to minimize PTX-binding to non glass surfaces. All solutions used in two microelectrode voltage clamp experiments had a pH of 7.4 ± 0.05 and osmolality of approximately 200 mOsm/kg.

Steady-state Patch Clamp measurements

HeLa cells were transiently transfected with rat $NK\alpha_1/NK\beta_1$ *c*DNAs encoding for rat Na,K-ATPase, rat $HK\alpha_2/NK\beta_2$ *c*DNAs encoding for rat colonic ngH,K-ATPase, or rat $NK\beta_1$ *c*DNA encoding for rat Na,K-ATPase β_1-subunit alone. Two days later, the cells were seeded on polylysine-coated coverslips and 10 μM ouabain was added to culture medium in order to inhibit endogenous Na,K-pumps. The cells were then incubated at 37°C in a 5% CO_2 atmosphere until confluence. The day after achieving confluence, steady-state patch clamp currents were measured at room-temperature (22-25°C). The patch pipette (1-3 MΩ) was filled with an intracellular solution containing (in mM): 85 Na-sulfamate, 20 TEA-Cl, 3 $MgCl_2$, 5.5 dextrose, 10 EGTA, 10 HEPES, 5 Na-pyruvate, 10 MgATP, and 7.9 phosphocreatine di-sodium salt. The K^+-free bathing solution contained (in mM): 145 NaCl, 23 $MgCl_2$, 2 $BaCl_2$, 5.5 dextrose, 10 HEPES/Na, and 0.2 $CdCl_2$. The solution containinig 20 mM K^+ to activate the Na/K pump was the same as the K^+-free solution but with equimolar replacement of NaCl by KCl. Ouabain (10 μM) was added to bathing solutions to inhibit endogenous sodium pumps before starting electrophysiological recordings. An aliquot of 100 μM PTX (Sigma) was thawed just prior to each experiment and diluted to a final concentration of 100 nM in the external K^+-free solution containing 0.002% BSA. All internal and external solutions used for patch clamp measurements had a pH of 7.4 ± 0.05 and osmolality of 280-300 mOsm/kg.

Direct PTX-application to confluent HeLa cells

The effect of palytoxin on cells over-expressing rat ngH,K-ATPase and Na,K-ATPase was studied in HeLa cells grown in 35 mm^2 Petri dishes to 50-60% confluency and then transiently transfected with rat $NK\alpha_1/NK\beta_1$ *c*DNAs encoding for Na,K-ATPase, rat $ngHK\alpha_2/NK\beta_2$ *c*DNAs encoding for (colonic) ngH,K-ATPase, or rat $NK\beta_1$ *c*DNA encoding for Na,K-ATPase β_1-subunit. Thirty six hours later, we treated all Petri dishes with 20 μM oubain in 1 ml culture media for 30 minutes to inhibit endogenous HeLa Na,K-ATPase. We then added 1 μM PTX to each Petri dish and the cells were incubated for 90 minutes at 37°C, 5% CO_2 atmosphere. Photographs were taken with a digital camera under phase contrast illumination at magnifications of 100X, 250X, and 400X. Petri dishes were photographed with phase contrast illumination at 250X and 400X.

Modeling of rat Na,K-ATPase and rat colonic H,K-ATPase

Modeller version 8.2 (Marti-Renom, et al. 2000) was used to create structural models of rat colonic ngH,K-ATPase and rat Na,K-ATPase based on a template of SERCA (Toyoshima, et al. 2004) in the E2-P conformation (PDB ID: 1WPG). Modeller's SALIGN command was used to construct a global, multiple alignment that included sheep Na,K and rat gastric H,K sequences to provide a consensus alignment in regions of lower identity. SERCA and rat ngH,K-ATPase share about 31% identity (Wang and Takeyasu 1997) but there is a much higher similarity in the intracellular domains as well as in the transmembrane sections where ion binding and permeation occur. All images were prepared with the PyMOL program (Delano, 2002. http://www.pymol.org) which is a molecular graphics system with a Python interpreter designed for real-time visualization and generation of high quality molecular graphics images.

Results

Functional Expression of Na,K-ATPase and ngH,K-ATPase

We carried out ^{86}Rb uptake measurements to test whether Na,K- and ngH,K-ATPase proteins expressed in HeLa cells and in *Xenopus* oocytes are functional. As shown in Fig. 1A, co-expression of rat Na,K-ATPase α_1 and β_1 or rat colonic ngH,K-ATPase α_2 and rat Na,K-ATPase β_2 in HeLa cells resulted in significant increases of ^{86}Rb uptake compared to cells expressing rat Na,K-ATPase β_1 or β_2-subunits alone. HeLa cells expressing rat Na,K-ATPase gave an increase of 8.8 ± 0.2 (n=6) fold over the background ^{86}Rb uptake measured in cells transfected with rat β -subunit alone. Inhibition of Na,K-ATPase by 10 mM ouabain gave background ^{86}Rb uptake similar to the controls with β_1 and β_2-subunits alone. Neither the ^{86}Rb uptake mediated by Na,K-ATPase nor that mediated by ngH,K-ATPase was affected by application of 10 mM SCH-28080 suggesting that neither is sensitive to this high dose of this compound. HeLa cells expressing rat ngH,K-ATPase displayed a ^{86}Rb uptake increase of 6.9 ± 0.3 fold over the ^{86}Rb uptake background measured in HeLa cells transfected with β_2-subunit alone. Application of 10 mM ouabain reduced ^{86}Rb uptake mediated by rat ngH,K-ATPase by about one-fifth consistent with a moderate sensitivity of rat ngH,K-ATPase to ouabain. Results of ^{86}Rb uptake studies in Na^+-loaded oocytes are shown in Fig. 1B. Oocytes expressing either *Bufo* bladder ngH,K-ATPase or *Bufo* Na,K-ATPase exhibited increases of 4.09 ± 1.04 fold and 4.35 ± 0.57 fold over ^{86}Rb uptake in oocytes injected with *Bufo* β_2-subunit alone. These results confirm that ngH,K- or Na,K-ATPase expressed in HeLa cells and in *Xenopus* oocytes are capable of significant ^{86}Rb uptake and, therefore, are expressed in the surface membrane as functional pumps. Additionally, the data in Fig. 1 are consistent with a moderate sensitivity of rat colonic ngH,K-ATPase to ouabain and its resistance to SCH-28080.

Palytoxin produces morphological changes on confluent HeLa cells expressing Na,K-ATPase

The effect of palytoxin could be directly observed as morphological changes produced in HeLa cells expressing Na,K-ATPase but not in those expressing ngH,K-ATPase. HeLa cells were treated with 20 μM ouabain (to inhibit endogenous Na,K-pumps) 30 minutes prior to PTX application. To visualize the changes in morphology that occurred after application of PTX, 1 μM PTX was added to the culture medium. After exposure to PTX (90 min), the medium was replaced by the solution used for electrophysiological measurements. Examination with phase contrast microscopy of cells expressing rat Na,K-ATPase showed that many clusters of cells had detached from the substrate, and were freely floating in the medium (Fig. 2A). These detached cells were small and round compared to attached cells that were spread flat against the substrate. Examination of the cells, that remain attached to the surface (at 400X) revealed granulations within their cytoplasm (Fig. 2B). The middle and lower rows in Fig. 2 show photographs of Petri dishes of cells expressing rat ngH,K-ATPase (C and D) or transfected with rat Na,K-ATPase β_1 subunit alone (E and F). Only a few small rounded cells are found that are freely floating in the medium. Most cells were flat and adherent to the substrate. Examination of these cells at 400X magnification showed that they were confluent and did not have cytoplasmic granulations (Fig. 2 D and F).

Effect of palytoxin on oocytes and HeLa cells expressing Na,K- and ngH,K-pumps

The two-microelectrode voltage clamp technique was used to measure currents generated by K^+-activation and PTX application in *Xenopus* oocytes expressing *Bufo* Na,K-ATPase, *Bufo* ngH,K-ATPase, or those injected with Bufo Na,K-ATPase β_2-subunit alone (Fig. 3A). An oocyte expressing *Bufo* Na,K-ATPase (top trace) was activated by 10 mM K^+ and generated a small outward Na,K-pump current (~0.2 μA). Exposure of oocytes expressing ngH,K-ATPase (middle trace) or those injected with β-subunit alone (bottom trace) to 10 mM K^+ did not produce this small initial outward current. A small inward current was generated after 10

mM K^+ activation of *Bufo* bladder H,K-ATPase. After returning to K^+-free solution measurements of membrane conductance were performed by making 50 mV depolarizing voltage steps from the holding potential of -50 mV at intervals of 30 s (vertical current excursions). Application of 5 nM PTX to the oocyte expressing *Bufo* Na,K-ATPase resulted in an in inward current and an increase of membrane conductance. The conductance increase was very large, on average up to 30 times the base line membrane conductance (Fig. 3B middle column). Oocytes expressing *Bufo* ngH,K-ATPase or those injected with β_2-subunit alone did not produce an inward current after 1 minute exposure of 5 nM PTX and the membrane conductance remained at the base line levels. Similar results were obtained at 10 nM PTX (data not shown). Results at 5 and 10 nM PTX from 8 to 10 oocytes were combined and are summarized in Fig.3B.

We also measured K^+-activated currents and the effect of PTX in HeLa cells using the whole-cell patch clamp technique at -40 mV (Fig. 4). The current traces in Fig. 4 illustrate activation of an outward Na,K-pump current on application of 20 mM K^+ in a cell expressing rat Na,K-ATPase (Fig. 4A, top trace), but not in cells expressing either ngH,K-ATPase (Fig. 4A, middle trace) or transfected with β_1-subunit alone (Fig. 4A, lower trace). After returning to K^+-free solution all three categories of cells were exposed to 100 nM PTX and the membrane conductance was measured after about 3 min of exposure. The membrane conductance of HeLa cells expressing rat Na,K-ATPase and exposed to PTX averaged 2.69 ± 0.24 nS; ~30 fold greater than the membrane conductance measured in HeLa cells exposed to PTX and expressing rat ngH,K-ATPase (0.095 ± 0.03 nS, n=6) or those transfected with β1-subunit alone (0.05 ± 0.01 nS, n=4). These results are summarized in Fig. 4B.

Rat ngH,K-ATPase and rat Na,K-ATPase models

If the failure of PTX to increase the conductance of ngH,K-ATPase is a result of failure to bind to the protein, we would expect to find significant structural differences in the extracellular domains of ngH,K-ATPase and Na,K-ATPase that could account for this difference. In order to determine if there are structural differences beween ngH,K-ATPase and Na,K-ATPase that could be related to PTX binding, we constructed structural models of rat Na,K- and rat (colonic) ngH,K-ATPase by alignment with SERCA. The alignments obtained correspond well with other alignments of type II_C-ATPases with SERCA (Munson, 2005, Rakowski, 2003). These models are based on the known crystal structure of SERCA and were constructed for E2-P conformation (PDB ID: 1WPG) (Toyoshima, et al. 2004) as described in Methods. The N-termini in type II_C ATPases are, in general, regions of low sequence identity. Indeed, Fig.5 shows a clear difference between the long N-terminus (green) of the rat Na,K-ATPase model and the short one (also green) of rat non-gastric H,K-ATPase. The Na,K-ATPase N-terminus is in a close proximity to the actuator domain, whereas the non-gastric H,K-ATPase N-terminus is hardly in contact with the actuator domain. Fig.5 also shows differences of the shape of the protruding M1-2 loop that may account for differences in ouabain and/or PTX binding affinity.

Discussion

The present study was designed to determine if PTX has an effect on non-gastric H,K-ATPases (rat colonic and *Bufo marinus* bladder) in cells in which they can be functionally expressed (*Xenopus* oocytes and HeLa cells) under experimental conditions in which the participation of endogenous Na,K-ATPase can be prevented by prior application of a low dose of ouabain. The data in Fig. 1 demonstrate that exogenous ngHK-ATPase and ouabain resistant NaK-ATPase expressed in HeLa cells and oocytes are functional as measured by ^{86}Rb uptake in cells in which endogenous NaK-ATPase activity is blocked by 10 μM ouabain. A clear and simple demonstration of the effect of PTX on the morphology of confluent HeLa cells over-expressing ouabain-resistant rat Na,K-ATPase is shown in Fig. 2A and B. After exposure to PTX the cells

can no longer maintain their normal gradients of electrolytes. The cells develop intracellular granulations, become more rounded as they swell, detach from the substrate and adjacent cells and eventually shrink to small spheres floating freely in the medium presumably because they lose their cellular contents across leaky surface membranes. HeLa cells expressing ngH,K-ATPase (Fig. 2 C, D) or Na,K-ATPase β_1 subunit alone (Fig. 2 E, F) that have their endogenous Na,K-ATPase blocked by 20 μM ouabain were not similarly affected.

We also performed measurements of membrane conductance on *Xenopus* oocytes expressing *Bufo* Na,K-ATPase or *Bufo* (bladder) ngH,K-ATPase. The results in Fig. 3 showed that PTX produced a large increase of membrane conductance in oocytes expressing Na,K-ATPase but not in those expressing either ngH,K-ATPase or H,K-ATPase β_2 alone. Patch clamp experiments in HeLa cells (Fig. 4) showed that PTX produced a very large increase in conductance (~30 fold) in cells expressing Na,K-ATPase but no significant increase in conductance in cells expressing (rat colonic) ngH,K-ATPase or rat Na,K-ATPase β_1 subunit alone. We conclude from these studies that Na,K-ATPase is the target of PTX action but not ngH,K-ATPase.

Rat ngH,K-ATPase and rat Na,K-ATPase structural models

Figure 5 shows a marked difference between the N-termini of rat Na,K-ATPase and rat non-gastric H,K-ATPase models. This difference is due to a 40-residue shorter N-terminus in non-gastric H,K-ATPase than the one in Na,K-ATPase. The Na,K-ATPase N-terminus is situated close to the actuator domain, and that is thought to tilt the M1 helix by rotation of the A domain (Toyoshima, 2004). This change is thought to play a key role in the E1 to E2 conformational change of the enzyme. The absence of 40 residues in the H,K-ATPase N-terminus appears to reduce the interaction between the A domain and TM1. This is evident since the short alpha helix and strand that loop around the A domain in the Na,K-ATPase model are absent from the H,K-ATPase model. The Na,K-ATPase N-terminus has been shown to play a role in PTX-induced channel inactivation (Wu et al., 2003). The absence of these 40 residues in ngH,K-ATPase N-terminus may, therefore, account for the absence of a PTX effect on ngH,K-ATPase. Additionally, the TM1-2 extracellular loop, which is critical for the high affinity binding of ouabain (Sweadner and Donnet, 2001) protrudes towards the extracellular region in a different way in the non-gastric H,K-ATPase and Na,K-ATPase models. We suggest that this structural difference may account for the difference in sensitivity of ngH,K-ATPase and Na,K-ATPase to ouabain and or to PTX. Experiments that directly measure PTX binding to the two proteins are warranted to test if this is the explanation of the difference in their responsiveness. If differences in binding affinity are found, it should be possible to test which regions are involved in PTX and ouabain binding by constructing chimeras of the two ATPases.

It its well established that PTX binds to Na,K-ATPase and opens a conducting pathway through it. (Scheiner-Bobis, 1998; Wang and Horisberger 1997; Guennoun and Horisberger 2000, 2002; Hilgemann, 2003; Artigas and Gadsby 2003, 2004). Non-gastric H,K-ATPase has been extensively studied since the successful cloning of its α-subunit in 1992 (Crowson and Shull, 1992). There is controversy regarding its pharmacological sensitivity to ouabain (Codina et al. 1996; Cougnon et al. 1996; Cougnon et al. 1998; Sangan et al. 2000) and this complicates the interpretation of experiments in which both PTX and ouabain are present. It has been reported that PTX has an effect on both distal and proximal colon, and it has been suggested that this action of PTX is mediated by interaction with ngH,K-ATPase (Scheiner-Bobis et al. 2002). However, since the conductance increase produced by PTX action on the Na,K-ATPase is so large, the presence of only a small amount of Na, K-ATPase in distal and proximal colon would be sufficient to explain the results obtained in those tissues. For example, in oocytes in which endogenous Na,K-ATPase was blocked by 10 μM ouabain even very low levels of expression of cysteine-mutants of Na,K-ATPase resulted in a large increase of membrane conductance

upon exposure to 2-4 nM PTX (Guennoun and Horisberger 2000, 2002; Horisberger et al. 2004). Low levels of expression of Na,K-ATPase has been reported within the apical membranes of non-gastric cells (Gottardi and Caplan 1993) whereas, H,K-ATPases are present primarily at the apical surfaces (Caplan, 1997; Smolka, et al. 1983). We suggest therefore that the reported action of PTX on proximal and distal colon is due to the presence of Na,K-ATPase in those tissues even though mucosal tissue was treated with 1 mM ouabain prior PTX application. This apparently did not prevent the effect of PTX on the apical membranes. The effect of PTX on *Bufo* bladder H,K-ATPase (Guennoun et al., 2005) and on ATP1AL1, the Human ngH,KATPase (Grishin et al. 1994) was tested by electrophysiological measurements and no increase of membrane conductance was found with those H,K-ATPases (unpublished data). These results support the conclusion that PTX does not increase the conductance of non-gastric H,K-ATPases and that the conductance increase produced by PTX in various tissues is due to the presence of Na,K-ATPase.

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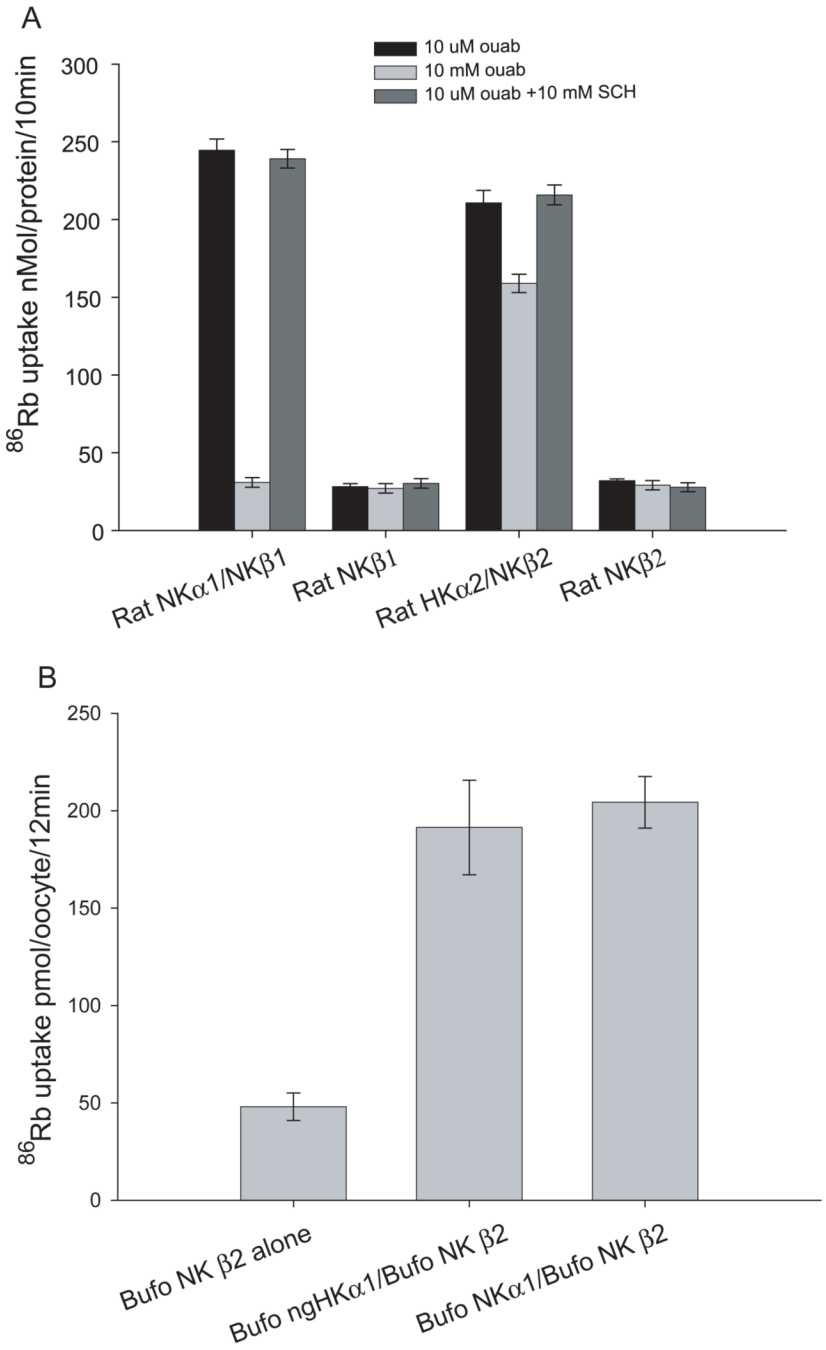

Figure 1.

(A) Measurements of ^{86}Rb uptake by HeLa cells. The four groups of 3 bars represent data obtained from HeLa cells transiently transfected with rat NKα_1/NKβ_1 *c*DNAs encoding for rat Na,K-ATPase (group 1 from the left), NKβ_1 *c*DNA encoding for rat Na,K-ATPase β_1-subunit (group 2), HKα_2/NKβ_2 *c*DNAs encoding for rat ngH,K-ATPase (group 3), and NKβ_2 *c*DNA encoding for rat Na,K-ATPase β_2-subunit (group 4). Assays for ^{86}Rb uptake (nMol/mg protein/10 min) were performed on each category of cells exposed to: a) 10 μM ouabain (black bars), b) 10 mM ouabain (light grey), and c) 10 μM ouabain plus 10 mM SCH-28080 (grey). The mean ± SEM is shown for 6 assays under each condition. HeLa cells expressing rat Na,K-ATPase or colonic ngH,K-ATPase exhibited increases of ^{86}Rb uptake of 8.8 ± 0.2 fold and 6.9

± 0.3 fold respectively over the ^{86}Rb uptake background observed with cells transfected with rat Na,K-ATPase β_1 or β_2 subunits alone. (B) Measurements of ^{86}Rb uptake by *Xenopus* oocytes. The mean ± SEM of^{86}Rb uptake (pMol/oocyte/12 minutes, 8-10 oocytes in each group) was measured in oocytes microinjected with *c*DNA encoding for *Bufo* Na,K-ATPase β_2-subunit alone (β_2)(left), *c*DNAs encoding for *Bufo* bladder H,K-ATPase and β_2 (middle), and *c*DNA encoding for *Bufo* Na,K-ATPase and β_2 (right). Oocytes expressing *Bufo* bladder H,K-ATPase and *bufo* Na,K-ATPase exhibited an increase of ^{86}Rb uptake of 4.09 ± 1.04 fold and 4.35 ± 0.57 fold respectively, over that measured in oocytes microinjected with β_2-subunit alone. Oocytes were Na^+ loaded prior to the uptake measurements. The solutions for uptake measurements in both A and B contained 10 μM ouabain to inhibit endogenous Na,K-pumps, and 10 μM bumetanide to block ^{86}Rb uptake mediated by the Na-K-2Cl co-transporter.

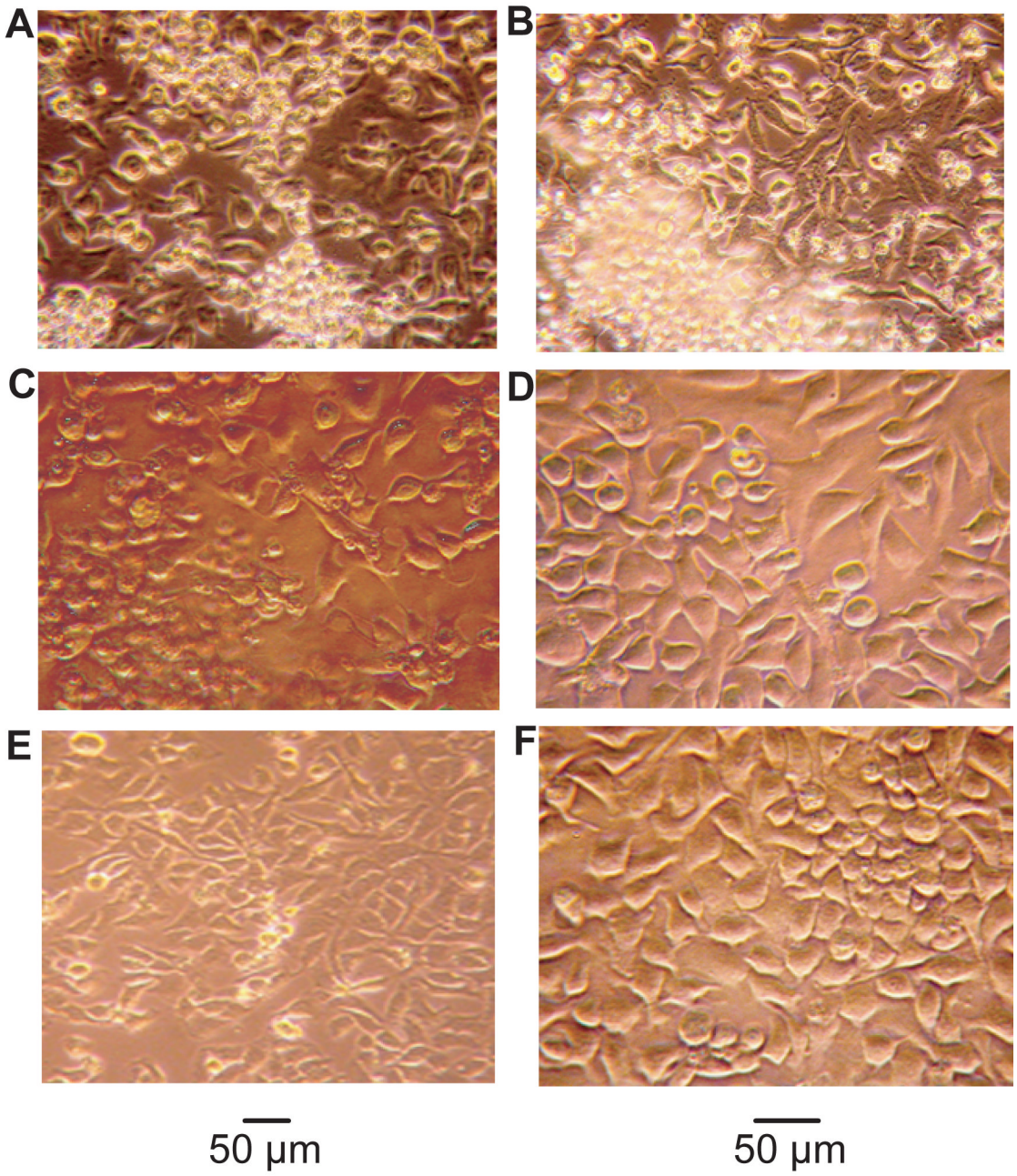

Figure 2.

Effect of PTX on confluent HeLa cells morphology. 1 µM PTX was applied for 90 minutes to 35 mm Petri dishes containing 1 ml of culture medium previously grown to confluence of HeLa cells transfected with rat $NK\alpha_1/NK\beta_1$ *c*DNAs encoding for rat Na,K-ATPase (Fig. 2A, 2B), rat $ngHK\alpha_2/NK\beta_2$ *c*DNAs encoding rat colonic ngH,K-ATPase (Fig. 2C, 2D), and $NK\beta_1$ *c*DNA encoding rat Na,K-ATPase β_1-subunit (Fig. 2E, 2F). Large clusters of cells expressing Na,K-ATPase (Fig. 2A and 2B) have swollen and detached from their neighboring cells and the substrate. They eventually become small, round and are found freely floating in the medium. Cells expressing rat Na,K-ATPase (Fig. 2B) show cytoplasmic granulations, whereas no granulations are seen within cells expressing ngH,K-ATPase (Fig. 2D) or β_1 subunit alone (Fig. 2F). Fig. 2A, 2C, and 2E were photographed with phase contrast illumination at 250X

magnification. Fig. 2B, 2D, and 2F were photographed with phase contrast illumination at 400X magnification. Petri dishes were treated with 20 μM ouabain for 30 minutes prior to PTX application. Four such experiments all showed similar morphological changes in cells expressing rat Na,K-ATPase and no similar changes were observed in cells expressing the rat colonic ngH,K-ATPase or those transfected with rat Na,K-ATPase β_1-subunit *c*DNA alone.

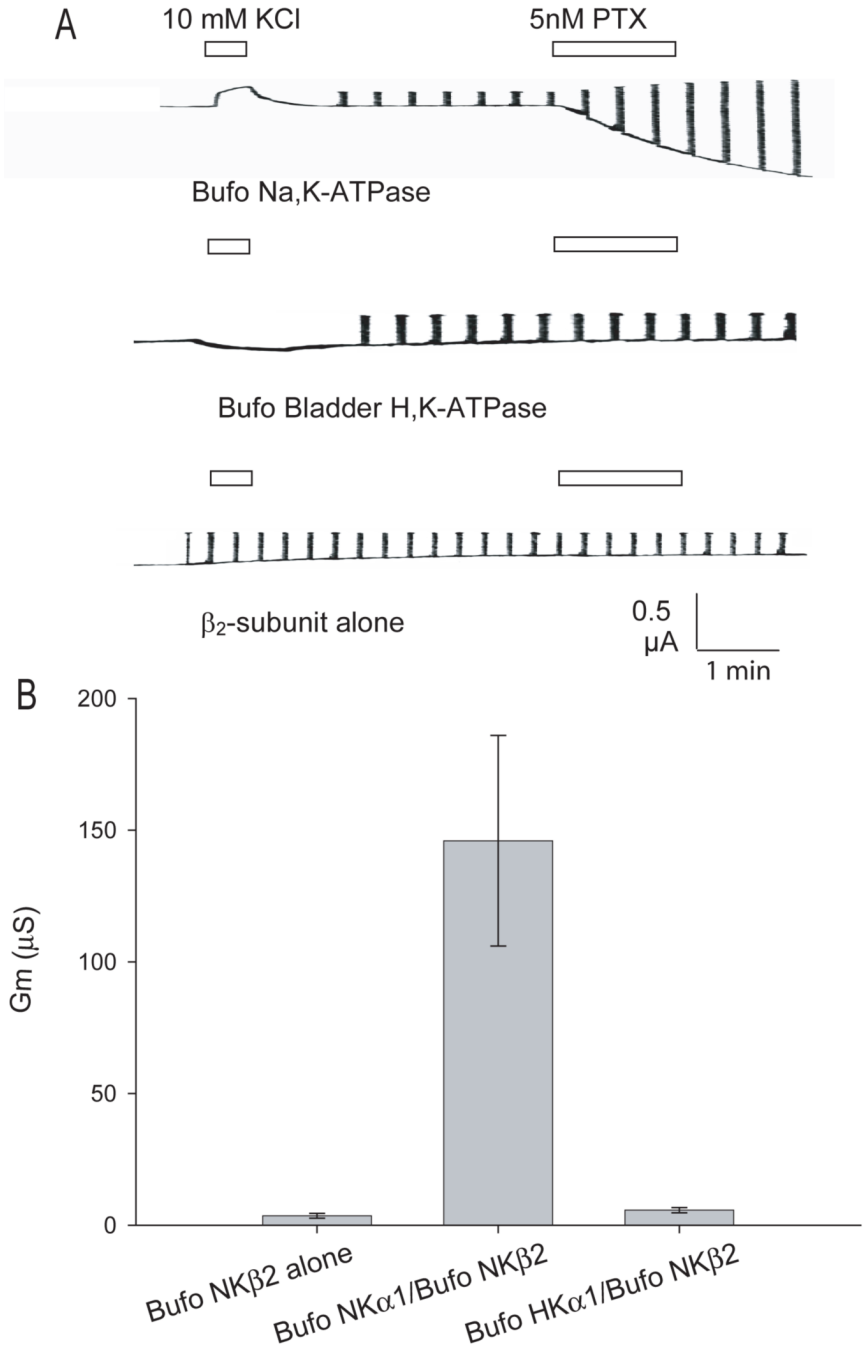

Figure 3.

Conductance changes produced by PTX in *Xenopus* oocytes. A, Representative traces of currents recorded using the two microelectrode voltage clamp technique in oocytes expressing *Bufo* Na,K-ATPase (top), *Bufo* bladder H,K-ATPase (middle), and *Bufo* Na,K-ATPase β_2-subunit alone (bottom). After clamping the oocyte at -50 mV in K^+-free solution (flow rate of 1 ml/min), solution containing 10 mM K^+ was applied for 30-60 s. Oocytes expressing Na,K-ATPase and exposed to 10 mM K^+ produced a small rapid increase in outward current (~0.2 μA) while oocytes expressing H,K-ATPase or those injected by β_2-subunit alone did not produce a response. PTX (5 nM) generated a large inward current that continued to increase

for several min after PTX removal in oocytes expressing *Bufo* Na,K-ATPase. No similar inward current was produced in oocytes expressing *Bufo* bladder ngH,K-ATPase or those injected with β_2-subunit alone. B. Palytoxin-induced conductance, G_m (μS) measured from oocytes injected with cRNA coding for *Bufo* Na,K-ATPase β_2-subunit (left column), *Bufo* Na,K-ATPase/β_2 (middle column), and *Bufo* bladder ngH,K-ATPase β_2 (right column). A large increase of membrane conductance occured in oocytes expressing *Bufo* Na,K-ATPase after exposure to 5 nM PTX (middle column). No significant membrane conductance changes were produced by 5 nM PTX in oocytes expressing ngH,K-ATPase or oocytes injected with β_2-subunit *c*RNA alone. The oocytes were exposed to 10 μM ouabain prior to electrophysiological measurements. Values are means ± SEM of 8-10 measurements.

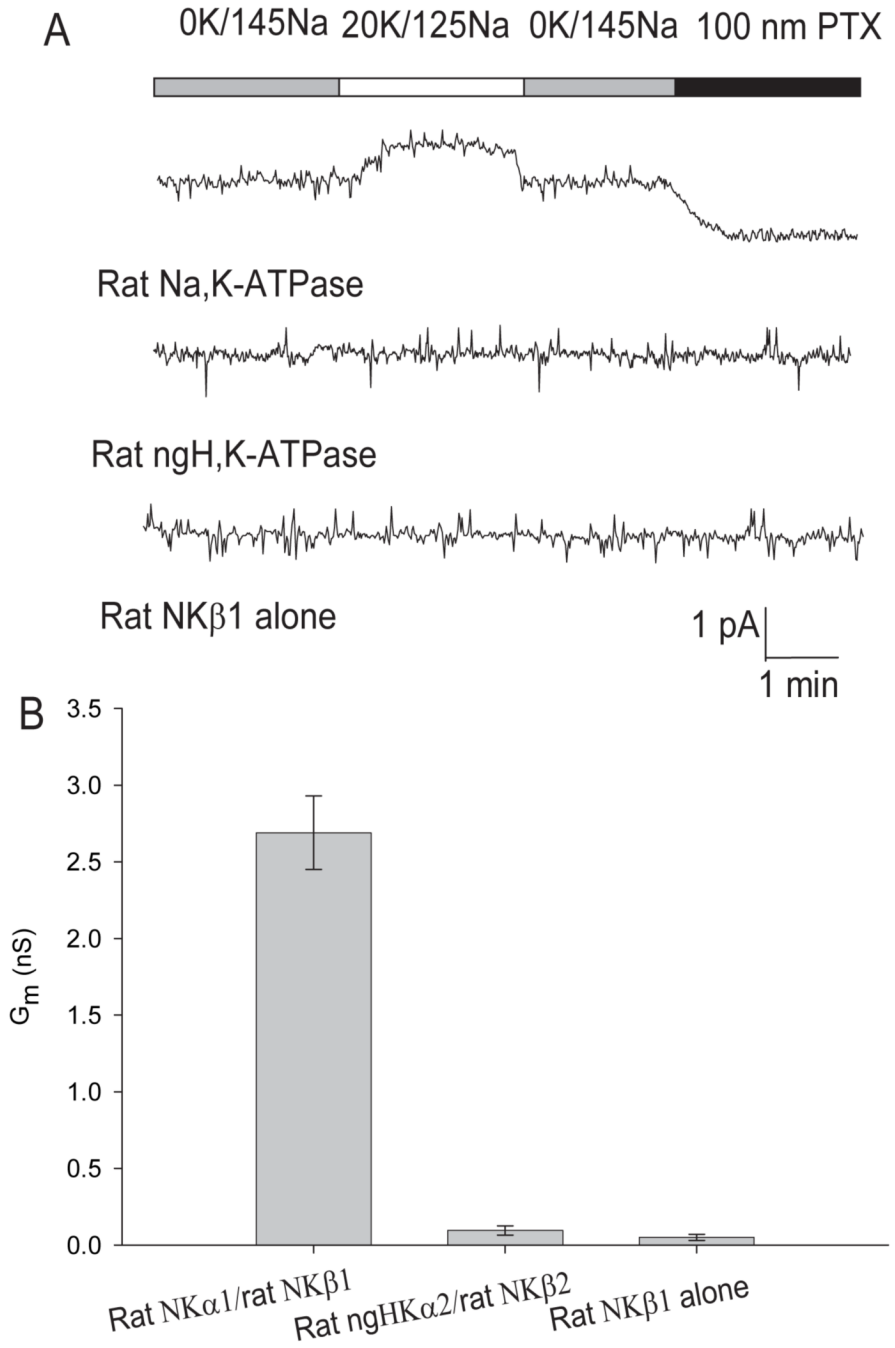

Figure 4.

Measurements of conductance changes produced by PTX in HeLa cells. A. Typical current traces obtained in whole-cell patch clamp experiments (-40 mV holding potential) in HeLa cells expressing rat Na,K-ATPase (top trace), ngH,K-ATPase (middle), or rat Na,K-ATPase β_1-subunit *c*DNA alone (bottom). Cells expressing Na,K-ATPase produced an outward current presumably mediated by the Na,K pump whereas cells expressing ngH,K-ATPase or rat Na,K-ATPase β_1-subunit alone did not show an immediate response to K^+ (middle and lower traces respectively). PTX application produced a large inward current in cells expressing rat Na,K-ATPase while no significant intward current after 3 min application of PTX to cells expressing rat ngH,K-ATPase or those transfected with rat Na,K-ATPase β_1-subunit *c*DNA. B. Mean

values of PTX-induced membrane conductance changes (G_m). Vertical bars indicate the mean values ± SEM of conductance changes measured from cells expressing rat Na,K-ATPase (left column), rat ngH,K-ATPase (middle column), and cells transfected with rat Na,K-ATPase β_1-subunit (right column). A large conductance was produced by PTX application on cells expressing rat Na,K-ATPase but no significant membrane conductance increase occurred after 3 min application of 100 nM PTX to cells expressing rat ngH,K-ATPase or transfected with rat Na,K-ATPase β-subunit.

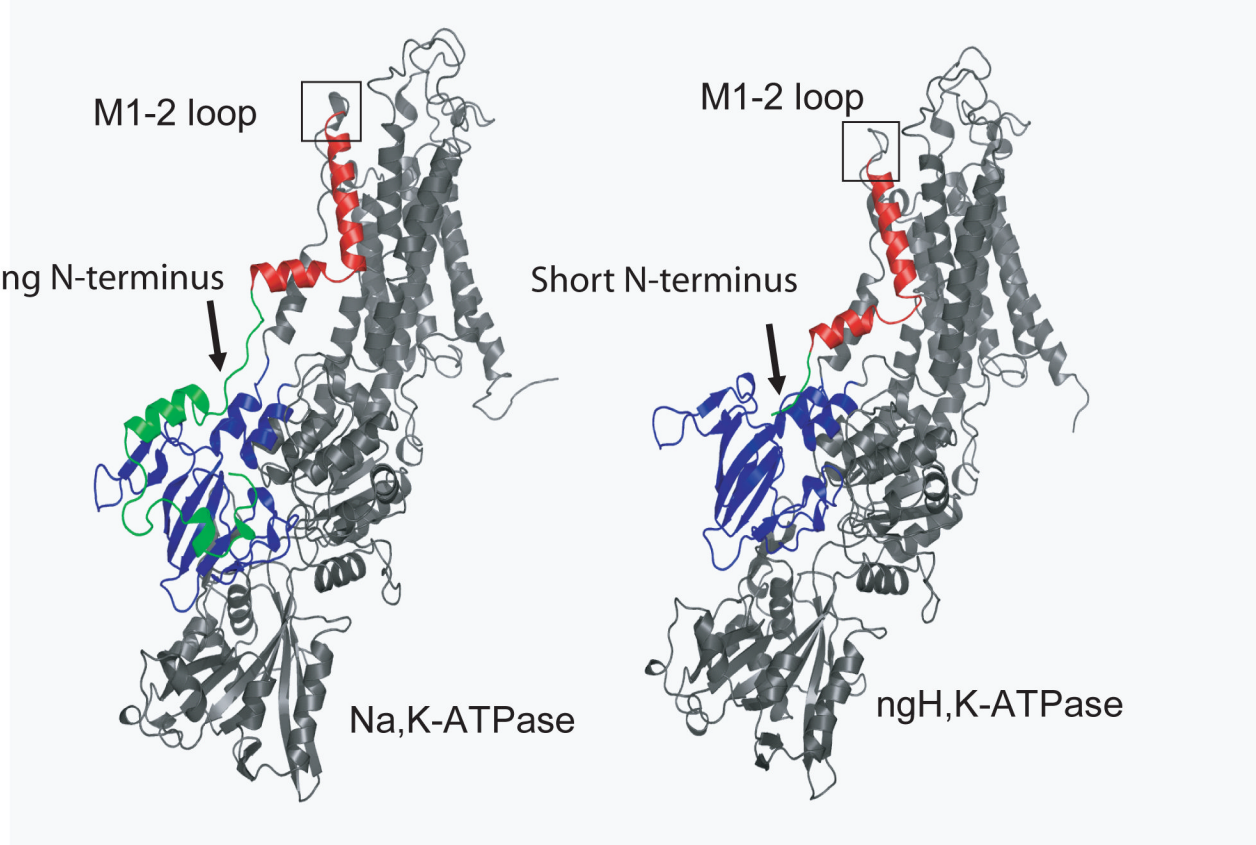

Figure 5.
Views of rat Na,K-ATPase (left) and rat ngH,K-ATPase (right) models based on E2 (PDB ID: 1WPG) crystal structures of SERCA. Transmembrane segments M1 are shown in red. The actuator domains are shown in blue and N-termini in green. The boxes indicate the M1-2 loops, and the arrows indicate N-termini. The remaining parts of the models are quite similar and are shown in grey.