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Divalent Cation Interactions with Na,K-ATPase Cytoplasmic Cation Sites: Implications for the *para*-Nitrophenyl Phosphatase Reaction Mechanism

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Abstract The interactions of divalent cations with the adenosine triphosphatase (ATPase) and *para*-nitrophenyl phosphatase (pNPPase) activity of the purified dog kidney Na pump and the fluorescence of fluorescein isothiocyanate (FITC)-labeled pump were determined. Sr^{2+} and Ba^{2+} did not compete with K^+ for ATPase (an extracellular K^+ effect). Sr^{2+} and Ba^{2+} did compete with Na^+ for ATPase (an intracellular Na^+ effect) and with K^+ for pNPPase (an intracellular K^+ effect). These results suggest that Ba^{2+} or Sr^{2+} can bind to the intracellular transport site, yet neither Ba^{2+} nor Sr^{2+} was able to activate pNPPase activity; we confirmed that Ca^{2+} and Mn^{2+} did activate. As another measure of cation binding, we observed that Ca^{2+} and Mn^{2+}, but not Ba^{2+}, decreased the fluorescence of the FITC-labeled pump; we confirmed that K^+ substantially decreased the fluorescence. Interestingly, Ba^{2+} did shift the K^+ dose-response curve. Ethane diamine inhibited Mn^{2+} stimulation of pNPPase (as well as K^+ and Mg^{2+} stimulation) but did not shift the 50% inhibitory concentration (IC_{50}) for the Mn^{2+}-induced fluorescence change of FITC, though it did shift the IC_{50} for the K^+-induced change. These results suggest that the Mn^{2+}-induced fluorescence change is not due to Mn^{2+} binding at the transport site. The drawbacks of models in which Mn^{2+} stimulates pNPPase by binding solely to the catalytic site *vs.* those in which Mn^{2+} stimulates by binding to both the catalytic and transport sites are presented. Our results provide new insights into the pNPPase kinetic mechanism as well as how divalent cations interact with the Na pump.

Keywords Na pump · Na,K-ATPase · *para*-Nitrophenyl phosphatase · Phosphatase · Calcium · Manganese · Barium · Strontium · Fluorescein isothiocyanate

Introduction

The kinetics of the related ion transport adenosine triphosphatases (ATPases) the Na pump, sarcoendoplasmic reticulum calcium ATPase (SERCA) and plasma membrane calcium ATPase have been extensively studied (*for reviews, see* Albers, 1967; Apell, 2003, 2004; Blostein, 1999; Horisberger, 2004; Jorgensen, Hakansson and Karlish, 2003; Kaplan, 2002; Martin, 2005; Moller, Juul and le Maire, 1996). These kinetic studies provide important insight into how these enzymes coordinate the translocation of ions with the hydrolysis of ATP. These studies have ruled out certain models of how these pumps work. The recent crystal structures of SERCA have answered some questions, clarified many points, constrained the models and raised new questions (Toyoshima, Nomura and Sugita, 2003, Toyoshima and Inesi, 2004).

In addition to ATP, the P-type pumps can hydrolyze other phosphate-containing substrates, such as *para*-nitrophenyl phosphate (Robinson, 1981, 1985; Robinson, Levine and Robinson, 1983; Drapeau and Blostein, 1980), 3-*O*-methylfluorescein phosphate (Huang and Askari, 1975) and acetylphosphate (Swann and Albers, 1980). A clear kinetic scheme has not been developed and accepted for this reaction, which complicates the analysis of several

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elegant mutagenesis studies (e.g., Vilsen, 1999) as well as the attempt to put together structural and kinetic features. One puzzle about the phosphatase reaction is whether it involves an occluded K^+ conformation. *Occlusion* is a state where the ion is trapped inside the protein and a conformational change is required in order to open up an access pathway and allow the ion to dissociate; i.e., when the ion is occluded, both the intracellular gate and the extracellular gate are closed.

Even though both *para*-nitrophenyl phosphatase (pNPPase) and dephosphorylation involve hydrolysis of a phosphate bond and both are stimulated by K^+, the side from which K^+ binds is different in the two cases. Extracellular K^+ stimulates E-P dephosphorylation (Blostein and Chu, 1977; Drapeau and Blostein, 1980). Intracellular K^+ stimulates pNPPase activity (Drapeau and Blostein, 1980), and it is this activity that is our focus here. In the presence of Na^+ and ATP, there is also a small pNPPase activity; in this case, it is stimulated by outside K^+ (Drapeau and Blostein, 1980). This Na^+-, ATP- and K^+-dependent pNPPase activity is easily accommodated in ATPase kinetic schemes and is not considered here.

A measure of Na pump conformations is the fluorescence of fluorescein isothiocyanate (FITC) (Karlish, 1980). FITC labels lysine-501, a key lysine in the nucleotide-binding domain (N domain) (Farley et al., 1984). The fluorescence of FITC at this site is sensitive to pump conformation (Karlish, 1980). When the transport site has internal access and is either empty or loaded with Na^+, one obtains maximal fluorescence. (Na^+ loading may have slightly higher fluorescence than the empty pump). When K^+ binds, the intracellular gate closes and K^+ becomes occluded. This triggers a change in the N domain which alters the environment surrounding FITC and produces a decrease in fluorescence. In the unlabeled pump, K^+ binding decreases the affinity of ATP binding, which presumably reflects the same link between intracellular gate closing and the N domain conformation.

Interestingly, even though the Na pump transports monovalent cations, divalent cations can bind to the transport site. There is at least one separate Mg^{2+} site which is important both for ATPase activity and for pNPPase activity (Karlish 2003), and K^+ and Na^+ can bind at that site (Robinson, 1981; Robinson and Pratrap, 1993). Therefore, we will use the term *transport site* to refer to where Na^+ or K^+ binds in the transmembrane domains and activates ATPase or pNPPase or changes pump fluorescence. The term *catalytic/hydrolysis site(s)* refers to where physiologically Mg^{2+} binds in the cytoplasmic domain and is involved in hydrolytic mechanisms. Na^+, K^+ and Mg^{2+}, at appropriate concentrations and conditions, can bind to the transport site, the catalytic site or both (Robinson, 1981; Robinson and Pratrap, 1993).

The divalent ion effects on pNPPase raise some puzzling questions. Huang and Askari (1984) have shown that Ca^{2+}, in the presence of Mg^{2+} but the absence of K^+, activates pNPPase activity. Two studies are consistent with this activation taking place at the transport site (Forbush, 1988; Vasallo and Post, 1986) since they find that Ca^{2+} is occluded. One difficulty with Ca^{2+} activation of pNPPase is that Ca^{2+} can go to both the catalytic site and the transport site but, while Ca^{2+}, presumably at the transport site, activates pNPPase, Ca^{2+} at the catalytic site is inhibitory. Because Ba^{2+} is closer to Ca^{2+} in size, here we characterize the interaction of Ba^{2+} with the pump.

Robinson (1981) has shown that Mn^{2+} supports pNPPase in the absence of Mg^{2+} and K^+. Robinson suggests that this is because Mn^{2+} favors the E2 conformation, but in other experiments he does allow that Mn^{2+} can bind to the transport site. Since Ca^{2+} seems to bind to the transport site and activates pNPPase, we examined whether Mn^{2+} stimulation could be simply Mn^{2+} at the transport site and not Mg^{2+}/Mn^{2+} selection for E1 vs. E2. (E1 and E2 have different but overlapping definitions. E1 can include states with the transport site accessible from the cytoplasm [and usually Na^+-loaded], with high FITC fluorescence, that can be phosphorylated with ATP, and that do not mediate pNPPase activity. E2 can include states with the transport site accessible from the extracellular medium or the transport site loaded with K^+ and inaccessible to either solution [occluded], with low FITC fluorescence, that cannot be phosphorylated with ATP and that do mediate pNPPase activity. E1 and E2 also have different protease sensitivities).

Here, we investigated divalent cation interactions with the Na^+ pump. We found that Ba^{2+} was able to go to the transport site but not to support pNPPase, in contrast to Ca^{2+}, which did. In addition, Ba^{2+} did not cause the decrease in fluorescence seen with K^+. While Mn^{2+} did cause the same decrease in fluorescence as K^+ and did support pNPPase activity in the absence of K^+, the results of ethane diamine inhibition suggested that the Mn^{2+} activation did not occur because Mn^{2+} bound to the transport site. These findings have implications for the structural basis of pump function and for our understanding of pNPPase activity.

Materials and Methods

Materials

[^{32}P]ATP was from Perkin-Elmer Life Sciences (Waltham, MA). Ammonium molybdate, hydrochloric acid, sodium phosphate, potassium chloride, sucrose, choline chloride, ascorbic acid, ß-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), imidazole, magnesium chloride, Tris

ATP, sodium bicarbonate, sodium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Trizma base were from Sigma (St. Louis, MO); $BaCl_2$, $SrCl_2$, $CaCl_2$ and $TbCl_3$ were from Fisher (Fairlawn, NJ). Dog kidneys were salvaged from euthanized dogs that were part of another approved IACUC, Institutional Animal Care and Use Committee protocol and were the generous gift of Dr. James L. Cook (University of Missouri-Columbia).

Na,K-ATPase Assay

Na,K-ATPase was purified from dog kidney as described previously (Jorgensen, 1974; Gatto et al., 2005). The ouabain-sensitive activity of the enzyme preparations used in this study ranged 7-14 μmol hydrolyzed ATP · mg protein^{-1} · min^{-1}. ATP hydrolysis was measured as reported previously for the red cell Ca^{2+} pump (Gatto et al., 2006) with minor modifications for Na,K-ATPase. Briefly, 0.1 mg quantity of purified canine renal sodium pump enzyme was diluted into 2.8 ml of 200 mM imidazole, titrated to pH 7.4 with dilute HCl. This mixture was warmed at 37°C for 10 min before being diluted 12-fold into 550 μl of an assay solution containing 50 mM imidazole at pH 7.4, 10 mM $MgCl_2$, 0.5 mM Tris ATP, 0.5 μCi [^{32}P]ATP, either 16 mM NaCl or 5 mM KCl, plus indicated concentrations of exogenous multivalent cations. Low [Na^+] was used to reduce the competition with the inhibitory cations. (Measurements at submillimolar ATP allow for a high ouabain-sensitive ATPase signal with low amounts of radioactivity; although 0.5 mM ATP is not saturating, the Na pump operates at >75% maximum velocity (V_{max}) with respect to [ATP] and these conditions are sufficient for determining whether multivalent cations compete with Na^+ or K^+). The reactions were stopped with the addition of 1 ml of an ice-cold solution containing 12 mM trichloroacetic acid and 100 g/l activated charcoal. The activated carbon binds organophosphates and is removed from the suspension by centrifugation in a microfuge at 4,000 rpm for 2 min. A 0.5-ml volume of each supernatant containing unbound inorganic phosphate was analyzed via liquid scintillation spectroscopy.

pNPPase Activity

All pNPPase assays were conducted essentially as described by Drapeau and Blostein (1980) using 2-4 μg of Na,K-ATPase from dog kidney preparations. It is important to note our observation that the pNPPase activity was sensitive to the total salt concentration of the reaction medium, especially below 100 mM total salt (*data not shown*). Thus, we were careful to match the concentration of inhibitor present with equal molar amounts of imidazole, Tris, 3-(*N*-morpholino)propanesulfonic acid (MOPS) or choline (we detected no differences between the ionic strength equalizing cations). In brief, the assay buffer contained 50 mM MOPS/Tris, 3 mM $MgCl_2$ (pH 7.4) with a final concentration of 5 mM di-Tris pNPP and 100 mM choline-Cl (or other salt for ionic strength equilibration) for each reaction tube. For 50% inhibitory concentration (IC_{50}) experiments, 2 mM K^+ was used while varying the concentration of tetrapropyl ammonium (TPA) over the range indicated in the figure legends. For K^+ competition experiments, the TPA concentration was fixed while varying the concentration of K^+ as indicated in the figure legends. Each reaction tube was incubated at 37°C for 15 min and the reaction stopped by addition of 200 μl of ''ice-cold'' 200 mM NaOH, with the reaction tubes placed in an ice bath for 10 min. Absorbance at 410 nm was then recorded utilizing a Beckman (Fullerton, CA) DU-530 spectrophotometer and converted to activity based on a pNP standard curve. We were careful to ensure that these experiments measured initial rates; i.e., <5% of the pNPP is hydrolyzed. Thus, free PO_4 concentrations never rose above 200 μM in these experiments and thus did not complicate conclusions based on inhibitor studies.

For Mn-stimulated pNPPase activity, we continuously measured the increase in pNP absorbance after addition of Mn^{2+} (in the absence of K^+ and Mg^{2+}). At intervals of approximately 1-2 min, ethane diamine was added. The slopes were determined at each ethane diamine concentration. For the temperature dependence of Mn-stimulated pNPPase activity, we measured phosphate release using the same assay as for ATP hydrolysis.

FITC Labeling and Fluorescence Measurements

We used a modification of the technique developed by Lin and Faller (2000). The Na pump was incubated with 10 μM FITC in 50 mM Tris HCl, 100 mM NaCl and 1 mM EDTA (pH 7.4) for 15 min at 37°C in the dark. The reaction was stopped by adding ~10 volumes of ice-cold 50 mM Tris HCl, 100 mM NaCl and 1 mM EDTA (pH 7.4) with 1 mM ß-mercaptoethanol and then dialyzed against the same buffer on ice for 30 min using a Pierce (Rockford, IL) Slidealyzer. A second dialysis on ice was done against 50 mM TrisHCl, 100 mM NaCl and 1 mM EDTA (pH 7.4) with 10 μM bovine serum albumin (BSA). Then, the solution was dialyzed against 50 mM Tris HCl, 100 mM NaCl and 1 mM EDTA (pH 8.0) with 10 mM dithiothreitol (DTT) at 37°C for 15 min and then continued for 15 min on ice. The DTT incubations were repeated two more times. After storage overnight, the solution was dialyzed against 50 mM Tris HCl, 1 mM EDTA (pH 8) until the DTT odor was removed.

The fluorescence emission spectra of the pump were determined using a Hitachi (Tokyo, Japan) F3100 Spectrofluorimeter in 200 mM *N*-methyl-D-glucamine (NDMG)

HEPES (pH 7.4), 0.5 mM EDTA and 150 mM tetramethylammonium chloride (TMA-Cl). Antifluorescein antibody was added until there was no further change of fluorescence. The peak fluorescence value was recorded and plotted as shown in the figures after addition of the appropriate compounds.

Results

The Na,K-ATPase preparation used for the experiments was purified from canine kidneys and treated with sodium dodecyl sulfate (SDS) (Jorgensen, 1974); consequently, this is an ''open'' preparation and all solutes have access to both intracellular and extracellular sites. As a test of whether the divalent cations bound to the extracellular transport site under these conditions, we examined whether they would compete with K^+ for Na,K-ATPase activity as this is an extracellular K^+ effect. As shown in Figure 1, Ba^{2+} and Sr^{2+} decreased the V_{max} of K^+ dependence of ATPase activity, which is not consistent with inhibition by these divalents at the extracellular transport site. To study inhibition at the intracellular transport site, high Mg^{2+} (i.e., 20 mM) concentrations were used to reduce the probability that divalent cations would bind at the catalytic site. Conversely, to study inhibition at the catalytic site, high concentrations of Na^+ or K^+ were used to reduce the probability that divalent cations would bind at the intracellular transport site.

The concentration dependence of divalent cation inhibition of Na,K-ATPase activity was measured (Fig. 2). Since our hypothesis was that these divalent cations would compete for binding to the cytoplasmic transport site, these experiments were performed with low Na^+ (i.e., 16 mM) in order to maximize inhibition. As expected, all three cations (Ba^{2+}, Ca^{2+} and Sr^{2+}) inhibited Na,K-ATPase activity.

If this inhibition by divalents occurs at the transport site, then it should be competitive with transported monovalent cations; e.g., it was expected that Ba^{2+} and Sr^{2+} would compete with Na^+ for activation of ATP hydrolysis and with K^+ for stimulation of pNPPase activity. Both divalent cations increased the Michaelis constant (K_m) for Na^+ but did not significantly alter V_{max} (Fig. 3) for ATPase activity. As shown in Figure 4, both Ba^{2+} and Sr^{2+} inhibited pNPPase activity by substantially increasing the K_m for K^+, whereas there was essentially no effect on V_{max}. These experiments were done at high Mg^{2+} concentrations to decrease the possibility of divalents binding to the catalytic site. Taken together, competition with Na^+ for ATPase and K^+ for pNPPase strongly supports the conclusion that these divalent cations bind at the intracellular transport site.

Since Ba^{2+} and Sr^{2+} bind to the intracellular transport site, we tested to see if they could activate pNPPase similar to K^+. First, we confirmed the observations that Ca^{2+} could stimulate pNPPase activity (Fig. 5). Similar to Askari's results (Huang & Askari, 1984), Ca^{2+} was not as effective as K^+, stimulating about 14% as well as K^+. In these experiments, Mg^{2+} is present; presumably, it is bound at the catalytic site and Ca^{2+} at the transport site.

We also confirmed Robinson's (1981) result that Mn^{2+} could stimulate pNPPase activity. As he reported, in con-

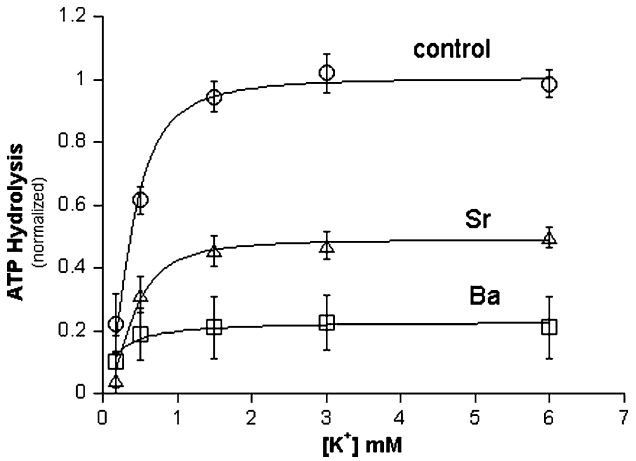

Fig. 1 Divalent cations do not compete with K^+ for ATPase activity. K^+ activation of ATPase activity was measured in the absence (○) and presence of 0.3 mM Ba^{2+} (□) or 0.9 mM Sr^{2+} (Δ). Increasing [K^+] did not overcome divalent cation inhibition of ATPase activity. Values were as follows: control, 1.00 ± 0.02; Ba^{2+}, 0.23 ± 0.01; Sr^{2+}, 0.49 ± 0.02. The K_m for K^+ was not altered by divalent cations. The data for each graph were fit to the Michaelis-Menten equation. Triplicate determinations from three separate experiments were normalized to the control V_{max} values. Points represent means ± SEM

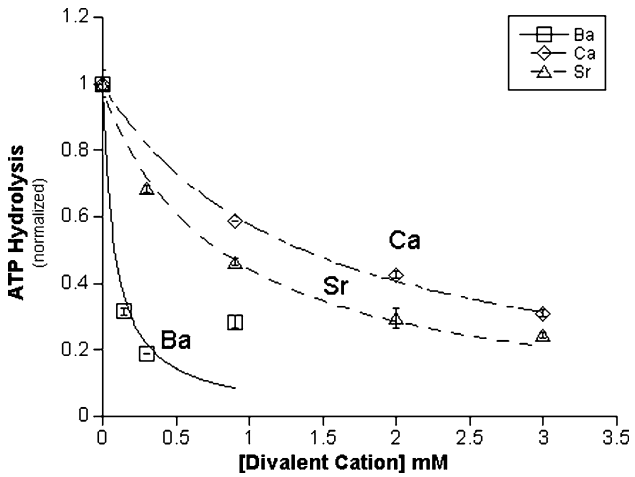

Fig. 2 Dose-dependent inhibition of Na,K-ATPase activity by divalent cations. Ouabain-sensitive ATPase activity of Na,K-ATPase purified from dog kidney was measured as described in Materials and Methods in the presence of the indicated concentrations of Ca^{2+}, Sr^{2+} and Ba^{2+} chloride. All three divalent cations produced a dose-dependent decrease in the rate of ATP hydrolysis, with Ba^{2+} being the most potent inhibitor. The IC_{50} values were as follows: Ba^{2+} = 0.08 ± 0.04 mM; Sr^{2+} = 0.8 ± 0.08 mM; Ca^{2+} = 1.3 ± 0.08 mM

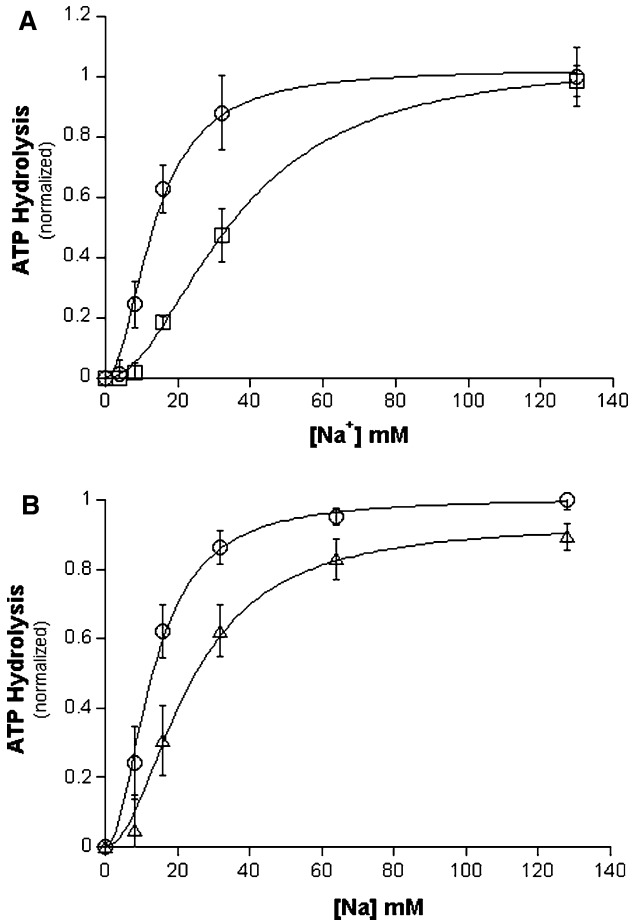

Fig. 3 Divalent cations compete with Na for ATPase activity. Na^+ activation of ATPase activity was measured in the presence of saturating substrates. Increasing concentrations of Na^+ in the presence of 0.3 mM Ba^{2+} (**a**) or 0.9 mM Sr^{2+} (**b**) decreased the inhibitory efficiency for both divalent cations, consistent with competition. Notably, the K_m for Na^+ increased by approximately 2.5-fold with divalent cations present compared to control K_m. V_{max} values were as follows: (**a**) control, 1.02 ± 0.03; Ba^{2+}, 1.06 ± 0.02; (**b**) control, 1.01 ± 0.02; Sr^{2+}, 0.93 ± 0.03

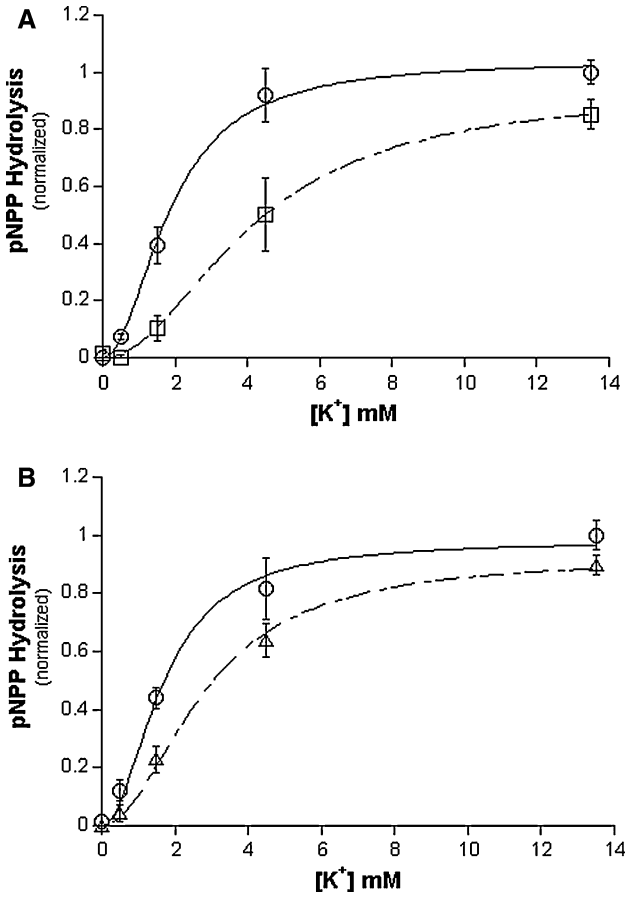

Fig. 4 Divalent cations compete with K^+ for phosphatase activity. The K^+ activation of pNPPase activity was measured in the presence of either 0.3 mM Ba^{2+} (**a**) or 3 mM Sr^{2+} (**b**). The presence of either divalent cation substantially increased the K_m for K^+ three- to fivefold, without significant altering V_{max}. The data were fit to the Michaelis-Menten equation, and resulting values were as follows: (**a**) control, V_{max} = 1.04 ± 0.02; K_m = 3.4 ± 0.3 mM; Ba^{2+}, V_{max} = 0.93 ± 0.02; K_m = 17.5 ± 1.1 mM; (**b**) control, V_{max} = 0.98 ± 0.04; K_m = 2.8 ± 0.5 mM; Sr^{2+}, V_{max} = 0.92 ± 0.03; K_m = 7.9 ± 1.0 mM. Triplicate determinations from three separate experiments were normalized to the control V_{max} values. Points represent means ± SEM

trast to Ca^{2+}, Mg^{2+} was not required for this stimulation (*data not shown*, *but see* Fig. 9).

In contrast to Mn^{2+} and Ca^{2+}, neither Ba^{2+} nor Sr^{2+} was able to activate pNPPase activity under our conditions (Fig. 5). These experiments were performed at pH 6.8, which Huang and Askari (1984) found to be the optimal pH for Ca^{2+}-dependent pNPPase activity. Thus, if there was any Ba^{2+}- or Sr^{2+}-stimulated pNPPase activity, it must have been <10% of the activity of Ca^{2+} (i.e., <2% of K^+-stimulated pNPPase).

However, it was conceivable that divalent cations could compete with Mg^{2+} to inhibit pNPPase activity, which could potentially mask their ability to mimic K^+ at the transport site and promote phosphatase activity. We examined this possibility by measuring the Mg^{2+} dependence of pNPP hydrolysis. In these experiments, we used high concentrations of K^+ to reduce the probability that the divalent cations would bind to the monovalent cation site. As shown in Figure 6, Ba^{2+} increased the K_m for Mg^{2+} activation of pNPPase activity and had essentially no effect on V_{max}, implying that under these conditions Ba^{2+} and Mg^{2+} compete; thus, Ba^{2+} can go to both the catalytic site and the intracellular transport sites depending on the concentration of other ions present. Therefore, we retested whether Ba^{2+} or Sr^{2+} could facilitate phosphatase activity in the presence of saturating Mg^{2+} (i.e., 20 mM) to eliminate inhibition at the Mg^{2+} site. Yet, even at this high [Mg^{2+}], neither Ba^{2+} or Sr^{2+} was able to stimulate pNPP hydrolysis (*data not shown*).

We determined which divalent cations could mimic K^+ in decreasing the fluorescence of the FITC-labeled Na pump. Ca^{2+} and Mn^{2+} are closer to Na^+ in size and Ba^{2+} is

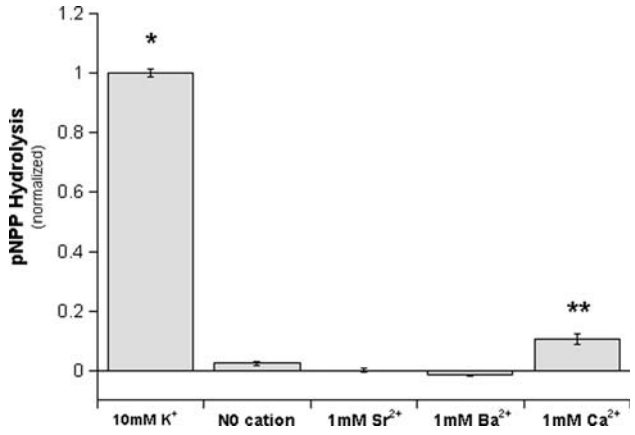

Fig. 5 Divalent cation support of pNPPase activity. Ouabain-sensitive pNPPase activity was measured on purified canine renal Na,K-ATPase in the presence of 1.5 mM Mg^{2+} and the indicated cation concentations. The data were normalized to the maximal activity observed in the presence of 10 mM K^+ (*bar 1*). The presence of Mg^{2+} (*bar 2*) sustained pNPP hydrolysis at ~4% of maximum. Neither Sr^{2+} (*bar 3*) nor Ba^{2+} (*bar 4*) could sustain measurable pNPP hydrolysis. In contrast, Ca^{2+} (*bar 5*) was able to sustain pNPP hydrolysis at ~14% of maximum. Triplicate determinations from three separate experiments were combined and plotted as the mean ± standard error. Analysis of variance was used to determine the difference between groups: *10 mM K^+ is different from all other groups, **1 mM Ca^{2+} is significantly different from *bars 2-4*

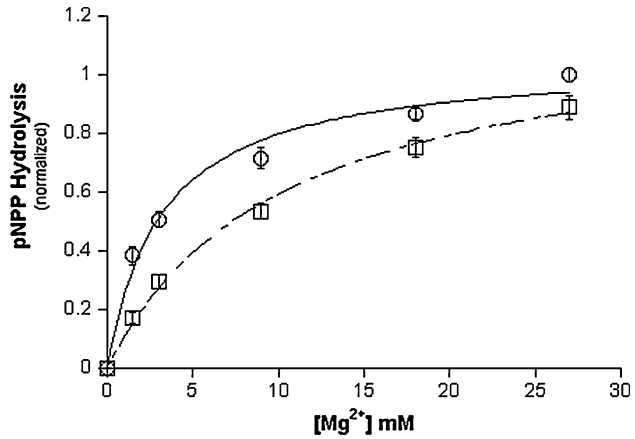

Fig. 6 Ba^{2+} competes with Mg^{2+} for phosphatase activity. The Mg^{2+} activation of pNPPase activity was measured in the presence of K^+ and the absence or presence of 1.2 mM Ba^{2+}. The presence of Ba^{2+} increased the K_m for Mg^{2+} threefold, without significant altering V_{max}. The data were fit to the Michaelis-Menten equation, and resulting values were as follows: control, V_{max} = 1.05 ± 0.06; K_m = 3.2 ± 0.7 mM; Ba^{2+} (□), V_{max} = 1.19 ± 0.07; K_m= 10.5 ± 1.5 mM. Triplicate determinations from three separate experiments were normalized to the control V_{max} values. Points represent means ± SEM

similar to K^+ in size, yet K^+, Ca^{2+} and Mn^{2+} supported pNPPase but Na^+, Ba^{2+} and Sr^{2+} did not. It was apparent that Mn^{2+} was able to mimic K^+ in causing a decrease in the FITC signal. The plateau of fluorescence is similar for both K^+ and Mn^{2+}, but the affinity for Mn^{2+} is lower than

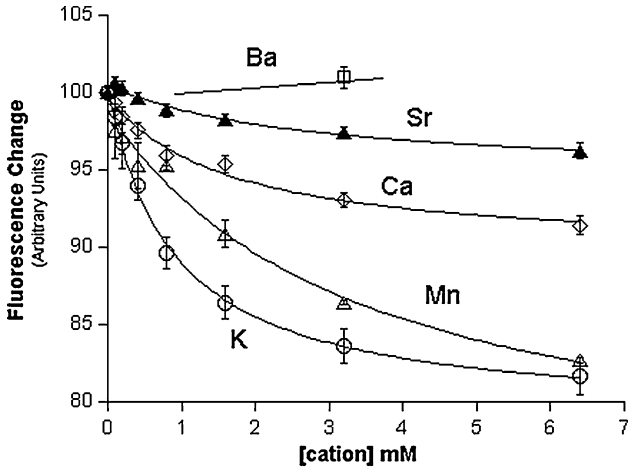

Fig. 7 Effect of divalent cations on FITC-labeled Na pump fluorescence. The fluorescence change in FITC-labeled Na,K-ATPase was monitored in the presence of increasing cation concentrations. Concentrations of Ba^{2+}, Sr^{2+}, Ca^{2+}, Mn^{2+} and K^+ were titrated into a cuvette containing FITC-labeled Na,K-ATPase, and the fluorescence (530 nm) was measured. A dose-dependent decrease in FITC fluorescence was induced by K^+, Mn^{2+} and Ca^{2+}, whereas Sr and Ba did not significantly decrease the fluorescence signal. The IC_{50} values for the cations that induced a K^+-like conformational change were as follows: K^+ = 0.9 ± 0.08 mM, Mn^{2+} = 3.3 ± 1.2 mM, Ca^{2+} = 1.6 ± 0.40 mM. Triplicate determinations from three separate experiments were normalized to the control V_{max} values. Points represent means ± SEM

that for K^+ (Fig. 7). Ca^{2+} also caused a decrease in FITC fluorescence; however, Ca^{2+} was not as effective as K^+ at reducing the FITC fluorescence signal (Fig. 7). In contrast, we observed no significant fluorescence reduction with either Sr^{2+} or Ba^{2+} (Fig. 7), consistent with their inability to stimulate pNPPase activity.

It could be argued that the lack of effect of Ba^{2+} on the FITC fluorescence could have reflected a very low affinity for Ba^{2+} binding to the pump under these conditions. However, from the pNPPase experiments (on unlabeled pump), we predicted that Ba^{2+} would bind at the transport site. Nevertheless, we tested directly whether Ba^{2+} could bind under these conditions; the effect of Ba^{2+} on the K^+ dose-response curve was determined. If Ba^{2+} cannot bind, then the K^+ dose-response curve should not change; but if Ba^{2+} does bind, then higher K^+ concentrations would be required. As shown in Figure 8, Ba^{2+} did indeed shift the K^+ response curve, consistent with Ba^{2+} binding to the pump under these conditions. Moreover, it confirms that Ba^{2+} cannot mimic K^+ in causing the conformational change that decreases FITC fluorescence or stimulates phosphatase activity.

The observation that Mn^{2+} stimulation of pNPPase occurs in the absence of Mg^{2+} (Robinson, 1981) could reflect Mn^{2+} binding to both the catalytic site and the transport site. However, Robinson suggested that Mn^{2+} stimulation oc-

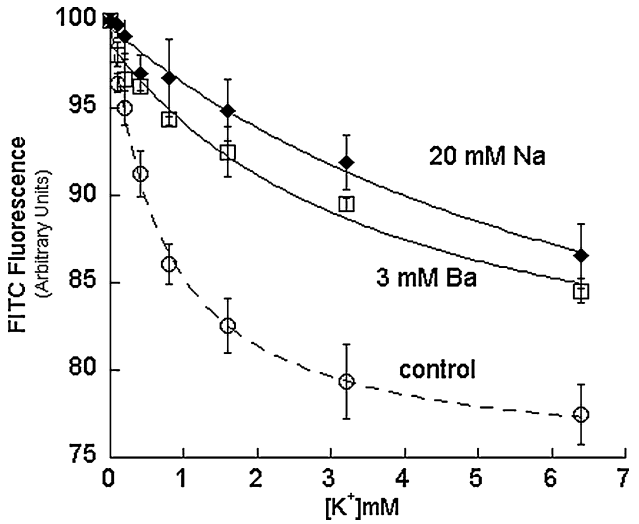

Fig. 8 Na^+ and Ba^{2+} increase the IC_{50} for K^+-induced decreased FITC fluorescence. The change in fluorescence of FITC-labeled Na,K-ATPase was monitored with increasing K^+ concentrations in the absence or presence of either 20 mM Na^+ or 3 mM Ba^{2+}. Clearly, the presence of either Na^+ or Ba^{2+} significantly increased the IC_{50} for K^+. The IC_{50} values for K^+ were as follows: K^+ alone = 0.7 ± 0.06, K^+ with Na^+ present = 8.2 ± 4.0, and K^+ with Ba present = 3.8 ± 1.6 Triplicate determinations from three separate experiments were normalized to the control V_{max} values. Points represent means ± SEM

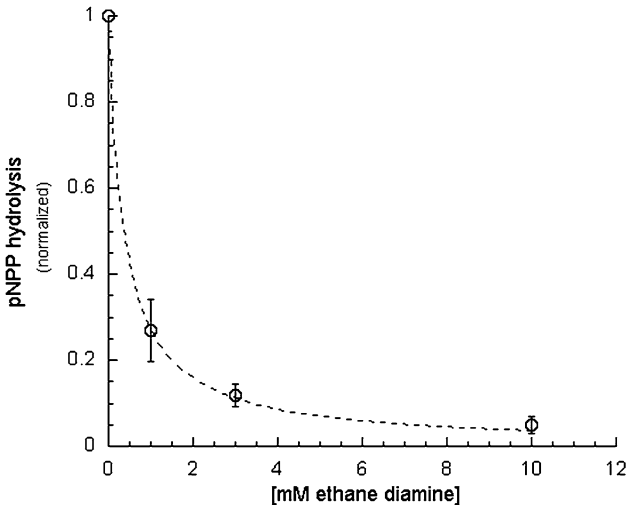

Fig. 9 Ethane diamine inhibits Mn-stimulated pNPPase. The Mn^{2+} activation of pNPPase activity was measured in the absence of K^+. The IC_{50} for ethane diamine inhibition was about 0.4 mM. Triplicate determinations from three separate experiments were normalized to the control values in the absence of inhibitor. Points represent means ± SEM

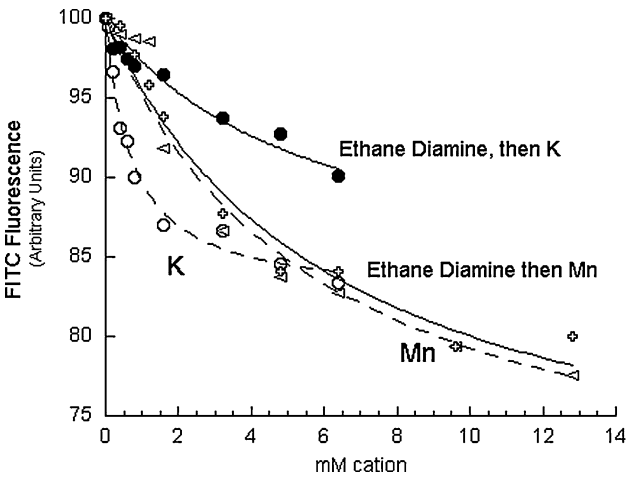

Fig. 10 Ethane diamine shifts the K, but not the Mn, response curves for cation-induced decreased FITC fluorescence. The change in fluorescence of FITC-labeled Na,K-ATPase was monitored with increasing K^+ or Mn^{2+} concentrations in the absence or presence of 10 mM ethane diamine. Clearly, ethane diamine significantly increased the IC_{50} for K^+ but did not change the IC_{50} for Mn^{2+}. Similar results were obtained in two other experiments

curred because Mn^{2+} favored E2 conformations more than Mg^{2+}. We wanted to determine if the Mn^{2+}-induced changes were due to Mn^{2+} binding at the transport site or the catalytic site. We examined the effect of ethane diamine, which binds from the cytoplasmic side and competes with K^+ for activation of pNPPase, on these Mn^{2+} responses.

Ethane diamine clearly inhibited Mn^{2+} stimulated pNPPase (Fig. 9). In contrast, ethane diamine did not alter the dose-response curve for Mn^{2+}-induced decrease in FITC fluorescence (Fig. 10). As a positive control, Figure 10 shows that this concentration of ethane diamine clearly shifted the K^+ response curve, even though K^+ has a higher affinity than Mn^{2+} for this response. Note that these FITC dose-response curves were done in the presence of pNPP to make them more comparable to the pNPPase conditions. These results suggest that the Mn^{2+}-induced change in FITC fluorescence is not due to Mn^{2+} being bound to the transport site. The other obvious site for Mn^{2+} to bind is the catalytic site.

In the next series of experiments, we examined the effect of Ba^{2+} on the Mn^{2+} response. Ba^{2+}, as well as ethane diamine, binds at the transport site (Fig. 3; Gatto et al., 2006). In contrast to ethane diamine, however, Ba^{2+} also binds at the catalytic site (Fig. 6). As shown in Figure 11, Ba^{2+} shifted the Mn^{2+} response.

Finally, we examined the effect of temperature on the affinity for Mn^{2+} stimulation of pNPPase because temperature alters the K_m at the transport site but not at the catalytic site (Robinson, 1989). We found that temperature had essentially no effect on the K_m for Mn^{2+} (*data not shown*).

Discussion

In this study, we examined the selectivity of different sites on the Na pump for divalent cations; the divalent ion

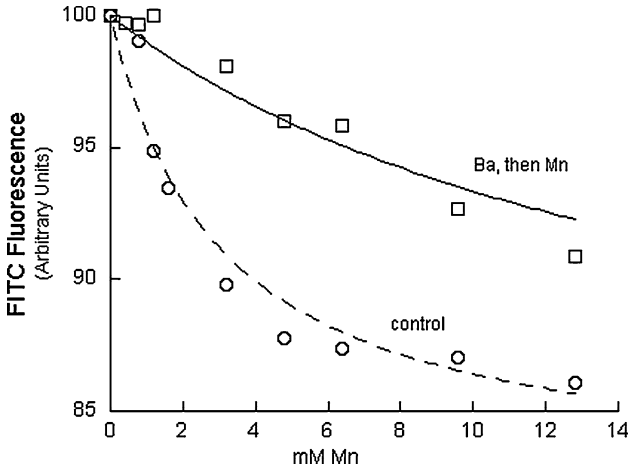

Fig. 11 Ba shifts the IC_{50} for Mn^{2+}-induced decreased FITC fluorescence. The change in fluorescence of FITC-labeled Na,K-ATPase was monitored with increasing Mn^{2+} concentrations in the absence or presence of 10 mM barium. Clearly, barium significantly increased the IC_{50} for Mn^{2+}. Similar results were obtained in at least three other experiments

effects on pNPPase activity and FITC fluorescence changes provide important new insights into the mechanism of pNPPase activity. We found the extracellular transport site excluded divalent cations. We found conditions where Ba^{2+} and Sr^{2+} bind exclusively to the intracellular transport site (e.g., high Mg^{2+}) or exclusively to the catalytic site (e.g., high intracellular Na^+ or K^+) but that even when Mg^{2+} is at the catalytic site, Ba^{2+} or Sr^{2+} binding to the transport site does not support pNPPase, in contrast to Ca^{2+}, even though Ba^{2+} and K^+ are similar in size. Furthermore, Ba^{2+} does not mimic K^+ in causing the decrease in FITC fluorescence, even though Ba^{2+} is bound, as evidenced by its ability to shift the K^+ dose-response curve. We also confirmed the observation that Mn^{2+} activates pNPPase in the absence of Mg^{2+} or K^+ and further demonstrated that Mn^{2+} mimics K^+ in causing a decrease in FITC fluorescence. All of these effects and the literature findings support a correlation between stimulation of pNPPase activity and inducing a K^+-like decrease in FITC fluorescence. (For example, Mn^{2+} does both, Ba^{2+} does neither, chymotrypsin eliminates both). However, we did find one condition where pNPPase activity and the decrease in FITC fluorescence were not correlated. Ethane diamine inhibited Mn^{2+}-activated pNPPase but did not alter the Mn^{2+} dose-response curve for the decrease in FITC fluorescence.

Relation to Previous Work

Schneeberger and Apell (2001) studied the interactions of divalent cations with the cytoplasmic transport site using RH421 fluorescence, which measures the electrogenic binding of the third Na^+. In the absence of Na^+, Ba^{2+} and Sr^{2+} appeared to have only unspecific effects at relatively high concentrations. However, Ba^{2+}, Sr^{2+}, Mg^{2+} and Ca^+ all shifted the Na^+ response curves. Specifically, 10 mM Ba^{2+} and Sr^{2+} increased the apparent K_m for Na^+ six- and 20-fold, respectively. Our results are in reasonable agreement with their data, particularly since RH421 and Na,K-ATPase measure different aspects of pump function. They also found that the Mg^{2+} response was physiologically relevant but more complicated than that for Ba^{2+} and Sr^{2+} (Schneeberger and Apell, 2001). The results are consistent with Mg^{2+} binding at both the catalytic site and the transport site.

Forbush (1988) and Vasallo and Post (1986) concluded that calcium can bind to the intracellular, but not the extracellular, transport site. Forbush (1988) directly measured $^{45}Ca^{2+}$ binding under conditions where the inside or the outside gate would be expected to be open. Vasallo and Post (1986) did not directly measure Ca^{2+} binding but reached the same conclusions. In our experiments, we found that Ca^{2+} was a mixed inhibitor of K^+-activated pNPPase activity. This is consistent with Ca^{2+} binding to two different sites: the transport site, where it competes with K^+, and the catalytic site, where it competes with Mg^{2+}. Others have previously shown that Ca^{2+} can go to the Mg^{2+} site (Beauge and Campos, 1983, 1986). Our data indicate that Ba^{2+} also goes to both sites. We feel the reason we can observe competitive kinetics with Ba^{2+} but mixed kinetics with Ca^{2+} relates to the relative affinities at the catalytic site for Mg^{2+}, Ca^{2+}, Ba^{2+} and K^+ as well as relative affinities at the intracellular transport site for K^+, Mg^{2+}, Ca^{2+} and Ba^{2+}. Thus, we were able to find conditions where Mg^{2+} was high enough to bind to the catalytic site but not so high as to prevent Ba^{2+} or K^+ binding to the intracellular transport site. In addition, we found a K^+ concentration where K^+ could bind to the intracellular transport but did not prevent Ba^{2+} or Mg^{2+} binding at the catalytic site. As far as we could determine, the Ca^{2+} affinity for the two sites was too similar to that of Mg^{2+} and K^+ to allow us to find a similar set of conditions for Ca^{2+}.

At What Site(s) Does Mn^{2+} Stimulate pNPPase?

K^+ and Ca^{2+} stimulation of pNPPase activity is thought to occur when either of these cations is bound to the transport site and Mg^{2+} is bound to the catalytic site. In contrast, Mn^{2+} can stimulate pNPPase activity in the absence of Mg^{2+}, which is not surprising since, in many systems, Mn^{2+} can replace Mg^{2+} (cf. Robinson, 1981; Campos and Beauge, 1988). We consider two models to explain the Mn^{2+} stimulation of pNPPase.

In model 1, Mn^{2+} binding at the catalytic site is sufficient to stimulate pNPPase activity. This model is similar

to Robinson's (1981) conclusion that Mn^{2+} activates pNPPase by favoring E2 conformations. In model 2, Mn^{2+} must bind to both the transport site and to the catalytic site in order to stimulate pNPPase activity.

We consider five key findings and how each model might fit with the data. For both models, in order to explain all the key data, additional modifications or explanations are required. However, we feel the explanations are more ''reasonable'' for model 1.

Model 1: Mn^{2+} binding at the catalytic site is sufficient to stimulate pNPPase activity

1. Mn^{2+} activation of pNPPase is hyperbolic (Robinson, 1981). In this model, only one Mn^{2+} needs to bind, so the curve is expected to be hyperbolic.
2. High Mn^{2+} inhibits pNPPase (Robinson, 1981). Since Mn^{2+} has only bound at the catalytic site for activation, it could be that at higher Mn^{2+} it binds to the transport site and at this site, Mn^{2+}, like Ba^{2+} and Sr^{2+}, inhibits.
3. Ba^{2+}, but not ethane diamine, shifts the Mn^{2+} dose-response curve for the change of FITC fluorescence (Figs. 10 and 11). In this model, Mn^{2+} is not bound at the transport site, so ethane diamine is not expected to have an effect on Mn^{2+} binding. While Ba^{2+} binds to both sites, it is Ba^{2+} preventing Mn^{2+} binding at the catalytic site that accounts for this effect.
4. Ethane diamine inhibits Mn^{2+}-stimulated pNPPase (Fig. 9). Ethane diamine binds only to the transport site and not the catalytic site. Since this model does not have Mn^{2+} binding to the transport site as essential to activation, ethane diamine inhibition cannot be explained by ethane diamine preventing Mn^{2+} from binding to the transport site. A reasonable possibility is that, with Mn^{2+} bound at the catalytic site, the pNPPase cycle includes a conformation where the inside gate to the transport site closes and ethane diamine prevents this gate from closing.
5. Mn^{2+} mimics K^+ in decreasing FITC fluorescence (Fig. 7). K^+ binding to the transport site leads to alteration of FITC fluorescence. This presumably occurs because when K^+ binds, a conformational change takes place, altering the environment of FITC, which is bound to the N domain. In this model, binding of Mn^{2+} to the catalytic site causes a similar conformational change to the N domain, and this is the structural analogy to Robinson's statement that Mn^{2+} causes the pump to be more E2-like than Mg^{2+}, which favors E1-type conformations. (E2 conformations, in general, support pNPPase and have decreased FITC fluorescence; E1 conformations do not support pNPPase and have increased FITC fluorescence).

Model 2: Mn^{2+} must bind to both the transport site and the catalytic site in order to stimulate pNPPase activity

1. Mn^{2+} activation of pNPPase is hyperbolic (Robinson, 1981). Since two Mn^{2+} ions must bind in this model, one must bind to one site with much higher affinity in order for the activation curve to be sigmoidal.
2. High Mn^{2+} inhibits pNPPase (Robinson, 1981). Since the two obvious sites are already filled for activation, Mn^{2+} must bind to yet another site or be a product inhibitor. There is no independent evidence for either of these possibilities.
3. Ba^{2+}, but not ethane diamine, shifts the Mn^{2+} dose-response curve for the change of FITC fluorescence (Figs. 10 and 11). This one is difficult to explain with this model.
4. Ethane diamine inhibits Mn^{2+}-stimulated pNPPase (Fig. 9). Ethane diamine inhibition of Mn^{2+}-stimulated pNPPase occurs simply because ethane diamine prevents Mn^{2+} binding at the transport site.
5. Mn^{2+} mimics K^+ in decreasing FITC fluorescence (Fig. 7). This occurs simply because both Mn^{2+} and K^+ binding at the transport site cause the same conformational change to the N domain that alters FITC fluorescence.

Implications for pNPPase Mechanism 1: Occluded Form and pNPPase

While there are several explicit kinetic schemes for the Na,K-ATPase cycle, there are no satisfactory schemes for the K^+-activated pNPPase cycle. Most investigators, including ourselves, if they draw a scheme, just include a vague conformation pump with K and pNPP bound (EK-pNPP).

We feel the existing data are most easily accommodated by a model in which the predominant conformation during pNPPase is occluded K^+ but the inside gate opens and closes (rapidly). Campos, Berberian and Beauge (1988) argued that the occluded state is not the major conformation. They showed that acid phosphatase deoccludes K^+ and indicated (but did not show) that pNPP does the same. Since inorganic phosphate and ATP also deocclude K^+, we think it is reasonable that any phosphate compound that binds to the pump is likely to speed up deocclusion. It is important to note that the method that Campos et al. (1988) used to measure deocclusion was by determining the release of radiolabeled Rb^+. We agree with their interpretation that pNPP causes the intracellular transport gate to open, thus allowing the radiolabeled ion to deocclude. Where we differ with them is that we think that this gate can close rapidly. Thus, in a bath containing K^+, the pre-

dominant conformation has both inside and outside gates closed and K^+ bound at the transport site between them. This K^+ can be exchanged rapidly; thus, depending upon how one defines ''occluded,'' it may or may not be an occluded state. In this model, it is the conformation with both inside and outside gates closed (and K^+ bound) that mediates pNPPase activity. Gonzalez-Lebrero et al. (2002) also indicate that, in the presence of ATP, the predominant form is pump with K occluded and ATP bound (E(K)ATP) but that the K^+ is rapidly released.

Implications for pNPPase Mechanism 2: FITC Fluorescence Changes and pNPPase

We think the existing data are consistent with the notion that during the pNPPase reaction the N and A domains are close and this causes the low fluorescence of FITC when K^+ is bound. The low fluorescence is due to the fact that either amino acids quench FITC or the local pH changes. This view is supported by the fact that chymotrypsin cuts the N and A connection and prevents both the FITC fluorescence change and pNPPase activity but the chymotrypsin enzyme is still able to occlude K^+ (Jorgensen and Petersen, 1985). There is a direct correlation between ions that support pNPPase and ions that change the FITC fluorescence: K^+, Mn^{2+} and Ca^{2+} do both, though Ca^{2+} does not give as large a change of fluorescence as K^+. Ba^{2+} neither supports pNPPase nor changes the fluorescence of FITC-labeled pump. Importantly, the Mn^{2+} effect on pNPPase is not due to Mn^{2+} binding at the transport site. Rather, we think Mn^{2+} straddles the N and A domains, similar to what has been observed for Fe^{2+} (Karlish, 2003).

We found that ethane diamine inhibits Mn^{2+}-activated pNPPase but not Mn^{2+}-induced FITC fluorescence. We offer two explanations for this result.

The first explanation is that the pNPPase cycle requires conversion between at least two2 conformations, A and B. These conformations have the same FITC fluorescence (e.g., the same abutment of N and A) but different states of the P domain. Ethane diamine prevents the change to conformation B and thus inhibits pNPPase, but this would be undetected by the FITC experiments. In model 1 above, conformation B requires the inside gate to close.

The second explanation also has at least two conformations, C and D. In this case, C and D have different FITC fluorescence levels. Furthermore, the change from C to D is rate-limiting for pNPPase such that most of the pump is in conformation C; thus, conformation C dominates the fluorescence signal. Ethane diamine inhibits by preventing the (brief) transition to D, so pNPPase cannot occur. The absence of ethane diamine shifts the amount of pump in conformation C from 98% to 99%, and this shift is undetectable with fluorescence. However, because this shift involves slowing the rate-limiting step for pNPPase, one can easily observe the inhibition of pNPPase activity.

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