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Kinetic characterization of Na,K-ATPase inhibition by Eosin

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Abstract

Eosin is a probe for the Na pump nucleotide site. In contrast to previous studies examining eosin effects on Na only ATPase, we examined Na,K ATPase and K activated pNPPase activity in red blood cell membranes and purified renal Na,K ATPase. At saturating ATP (3mM) the eosin IC_{50} for Na pump inhibition was 19uM. Increasing ATP concentrations (0.2 – 2.5 mM) did not overcome eosin-induced inhibition thus eosin is a mixed-type inhibitor of ATPase activity. To test if eosin can bind to the high affinity ATP site, purified Na,K ATPase was labeled with 20 uM FITC. With increasing eosin concentrations (0.1 uM – 10 uM) the incorporation of FITC into the ATP site significantly decreases suggesting that eosin prevents FITC reaction at the high affinity ATP site. Eosin was a more potent inhibitor of K activated phosphatase activity than of Na,K ATPase activity. At 5mM pNPP the eosin IC50 for Na pump inhibition was 3.8 ± 0.23 uM. Increasing pNPP concentrations (0.45 – 14.5 mM) did not overcome eosin-induced inhibition thus eosin is a mixed-type inhibitor of pNPPase activity. These results can be fit by a model in which eosin and ATP bind only to the nucleotide site; in some pump conformations, this site is rigid and the binding is mutually exclusive and in other conformations, the site is flexible and able to accommodate both eosin and ATP (or pNPP). Interestingly, eosin inhibition of pNPPase became competitive after the addition of $C_{12}E_8$ (0.1%) but the inhibition of ATPase remained mixed.

Introduction

The Na,K-ATPase (aka Na pump) is a nearly ubiquitous transmembrane protein in animal cells that actively counter-transports Na^+ and K^+ across the plasma membrane in a $3Na^+_{out}$ / $2K^+_{in}$ ratio, energized by the hydrolysis of one ATP molecule. The disequilibrium in ion transport makes the Na pump electrogenic. The Na^+ gradient created by the Na,K-ATPase is coupled to a multitude of secondary active transporters which mediate the uptake of amino acids, glucose, HCO_3^-, and neurotransmitters [1], as well as the extrusion of Ca^{++} and H^+ [2]. Moreover, the maintenance of the Na^+ and K^+ gradients, in combination with selective ion channels, gives rise to resting membrane potential in most animal cells [for review see 3,4]. Indeed, the homeostatic role of the Na pump is so critical that Na,K-ATPase activity accounts for approximately 23% of ATP hydrolysis in humans during rest [5] and is the major user of ATP in red blood cells from most species.

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The Na pump reaction cycle is conveniently described by a model proposed independently by Albers and Post [6,7], which describes two major conformations: the first has cation accessibility from the cytoplasm (E_{in}) and the second has cation access from the extracellular space (E_{out}). Briefly, the binding of 3 intracellular Na^+ ions shifts the pump to a conformation where it binds one molecule of ATP to a high affinity nucleotide binding site. The γ phosphate of ATP is transferred to the protein forming a phosphorylated intermediate E-P; this covalent phosphointermediate distinguishes P-type ATPases from the V-type and F_0F_1 ATPases [8]. A conformational shift accompanies the release of ADP and the translocation of Na^+ to the extracellular space. Two K^+ ions bind to the extracellular face and initiate the hydrolysis of the phosphoenzyme releasing the inorganic phosphate (P_i). A subsequent conformational change of the protein once again flips the cation binding site to the intracellular side and concomitantly releases K^+ ions. In the absence of K^+, the Na pump mediates a slower rate of ATP hydrolysis termed Na (only) ATPase activity. This Na (only) ATPase activity is activated by ATP with high affinity. The Na,K ATPase activity response to ATP shows both high and low affinity ATP effects; the low affinity ATP effect is thought to be the result of ATP stimulation of K^+ deocclusion. A kinetic model which generates both ATP effects but which requires only 1 structural ATP site remains the most popular description of the pump cycle (3, 4).

Fluorescein Inhibition

From the topology of the Na,K-ATPase, many of the initial studies regarding cation binding, ATP binding, and phosphorylation focused on the large cytoplasmic domain between transmembrane segments 4 and 5 (M4 and M5) of the α-subunit. This cytoplasmic segment is 430 amino acids in length of which, 180 residues comprise the ATP binding domain [9]. As hypothesized, the M4M5 cytoplasmic loop contains the nucleotide-binding site as evidenced by several studies utilizing chemical modification to inhibit ATP hydrolysis. It was first reported in 1980 that covalent modification with fluorescein 5′-isothiocyanate (FITC) abolished Na,K-ATPase activity in a 1:1 stoichiometry and that ATP protected against FITC labeling [10,11]. Nevertheless, the FITC-conjugated protein still bound inorganic phosphate and underwent Na^+ and K^+-induced conformational shifts [10]. Similarly, FITC inhibited ATPase activity in the sarcoplasmic reticulum Ca-ATPase in a 1:1 stoichiometry [12]. It was subsequently determined that FITC covalently attached to Lys^{501} of the canine Na,K-ATPase within a conserved sequence (i.e. **K**AG*P*/SER) [13]. The equivalent residue in SERCA (i.e. Lys^{515}) has been shown to be an ATP contact residue via crystallography [14].

Eosin, i.e.tetrabromofluorescein, is a reversible inhibitor that is competitive with ATP for the Na (only) ATPase [15]. Na (only) ATPase activity was measured in the absence of K^+ ions. Similarly, increasing ATP concentration overcame eosin inhibition of the gastric H,K-ATPase [16], consistent with eosin interacting with the ATP binding site. Indeed, the nucleotide site binding efficiency of eosin remains an exploited tool for real-time fluorescent monitoring of nucleotide binding site conformational changes [17,18,19]. Thus, it was surprising when we observed that eosin non-competitively inhibited ATP hydrolysis and ATP-dependent $^{45}Ca^{++}$ transport in the red cell plasma membrane Ca-ATPase [20]. Yet, like other P-type ATPases, FITC was an irreversible, ATP-protectable, inhibitor of PMCA, which modified the same conserved lysine residue (i.e. $\mathbf{K}^{591}$GASE) [21]. Consequently, it seems inappropriate to assume that eosin is an ATP site probe for all P-type ATPases in the absence of further experimentation.

In this study, we reexamined the inhibitory properties of eosin on Na,K-ATPase. Interestingly, we found that eosin appears to be a mixed-type inhibitor with respect to ATP for porcine red cell Na,K-ATPase and purified sheep kidney Na,K-ATPase. In addition, eosin did not compete with *para*-nitrophenylphosphate for K activated phosphatase activity in the kidney enzyme. However, when the purified renal Na,K-ATPase preparation was treated with a non-ionic

detergent (i.e. 0.1% $C_{12}E_8$), eosin did appear to be a mutually exclusive inhibitor of pNPPase activity. Our results show that eosin inhibition of the Na pump is more complicated than mere binding to the high affinity ATP site.

Materials and Methods

Materials

[^{32}P]ATP was from Perkin-Elmer Life Sciences. All other chemicals were reagent grade purchased either from Sigma (St. Louis, MO) or from Fisher Scientific (Pittsburgh, PA). Sheep kidneys and porcine red blood cells were purchased from Pel-Freez Biologicals (Rogers, AR). Purified Na,K-ATPase was obtained as described previously [22]. Red cell membranes were prepared as described previously [20]. Protein concentrations were determined by the method of Lowry et al. [23].

Na,K-ATPase Assay

Red Cell Na,K-ATPase activity was measured as reported previously for the red cell Ca pump [20], with minor modifications for Na,K-ATPase. Briefly, 0.5 mg of red cell membrane protein was diluted into 30 mL of assay media containing (in mM): 140 NaCl, 34 KCl, 33 HEPES (pH = 7.4), 4 $MgCl_2$, and 0.06 EGTA. Reactions tubes received 400ul of the above medium and were run at a total volume of 500ul in the presence or absence of 5uM eosin at the [^{32}P]ATP concentrations indicated in figure 1. Reactions were incubated at 37^{o}C for 80 min and stopped by adding 5mL of 5% TCA/charcoal. The charcoal was spun down in a microfuge and 175uL aliquots were taken from the supernatant and the liberated $^{32}PO_4$ determined via liquid scintillation spectroscopy. All tube were run in the presence and absence of 0.5mM ouabain and the differences were reported as the specific Na,K-ATPase activity.

Purified kidney Na,K-ATPase activity was measured as reported previously [24]. Briefly, Na,K-ATPase activity was measured in a standard assay medium containing (in mM): 1 EGTA, 130 NaCl, 20 KCl, 3 $MgCl_2$, 3 Na_2ATP, 50 imidazole (pH 7.2, 25oC), and 0.5 μg/ml purified enzyme. The suspension was incubated at 37^{o}C for 15 min and the liberation of PO_4 measured as described by Brotherus *et al.* [25]. The specific Na,KATPase activity was the difference between the ATP hydrolysis measured in the absence and presence of 0.5 mM ouabain.

para-nitrophenylphosphatase activity. All pNPPase assays were conducted essentially as described in [26] using 2-4ug of Na,K-ATPase from dog kidney preparations. Briefly, assay medium contained (in mM) 50 MOPS/Tris, 5 KCl, 3 $MgCl_2$ (pH 7.4) with a final concentration of 5mM di-tris pNPP and 100 Choline-Cl. For pNPP competition experiments, eosin concentration was fixed while varying the concentration of pNPP as indicated in the respective figure. Each reaction tube was incubated at 37^{o}C for 15 minutes and the reaction stopped by the addition of 200ul of “ice-cold” 200mM NaOH and the reaction tubes placed in an ice bath for 10 min. Absorbance at 410nm was then recorded utilizing a Beckman DU-530 spectrophotometer and absorbance was converted to activity based on a pNP standard curve.

Eosin protection against FITC labeling of the Na,K-ATPase

Na,K-ATPase (50 μg) was mixed with either 20 μM FITC (diluted from 10 mM stock in DMSO), 20 μM FITC and 5 mM ATP, or 20 μM FITC and eosin (concentration range, 0.1 – 10 μM, diluted from 10 mM stock in DMSO). A FITC concentration of 20 μM was chosen to reduce activity to less than 10% of controls. The reactions were incubate at room temperature for 30 minutes and stopped with 150 μl of ice-cold stopping solution (i.e. 50 mM Tris/100 mM NaCl/50 mM β–mercaptoethanol, pH 6.5). A 5 μg quantity of protein from each reaction was mixed with Laemmli sample buffer (1:1:1 v/v mixture of 8 M Urea, 10% SDS, 125 mM Tris Buffer) and loaded onto a 12% Laemmli gel [27]. The fluorescent protein bands were visualized

on a UVP Transilluminator Gel Documentation System with Image Store 7500 version 7.21 software. Aliquots from each FITC incubation were used to determine Na,K-ATPase activity as described above.

Results

The ATP-dependence of eosin inhibition of the red cell Na pump is shown in figure 1. In these experiments on red cell Na pump, the inhibition produced by 5uM eosin was not overcome by increasing ATP concentration. Similarly, increasing the ATP concentration from 0.1mM to 2.5mM also did not appear to diminish eosin inhibition of purified renal Na,K-ATPase (Fig. 2A). Clearly, the V_{max} in the absence of eosin (1.11 ± 0.02) was significantly different than either the V_{max} in the presence of 20uM (0.77 ± 0.02) or 50uM (0.53 ± 0.01) eosin.

The concentration dependence of eosin inhibition of purified Na,K-ATPase (sheep kidney) at saturating ATP shows that the eosin IC_{50} for Na pump inhibition was 19uM and is consistent with only 1 eosin binding site for inhibition. Interestingly, eosin is nearly 500-times more potent at inhibiting PMCA [20,28,29].

One test of whether eosin can bind to the high affinity ATP site is to see if it can prevent chemical modification of Lys-501 by FITC; lysine-501 is an ATP coordinating residue. We incubated purified Na,K-ATPase with 20uM FITC in the presence of eosin concentrations ranging from 0.1uM – 10uM (Fig. 3). The incorporation of FITC into the a-subunit significantly decreases with increasing [eosin] as one would predict if eosin bound to the ATP site. In addition, aliquots from the FITC-treated enzyme were used to measure ATPase activity to ensure that modification was at the critical lysine residue (Fig. 3B). When the eosin concentration is plotted against remaining activity, it reveals a K_{app} for eosin of 0.83 ± 0.06uM. The fact that the apparent affinity drops >20-fold in the absence of ATP is also consistent with the two sharing a common binding site. However, eosin and ATP binding simply binding to the same site would predict mutually exclusive binding, which is not what was observed (Figs. 1 and 2B).

We wanted to investigate the effect of eosin on an alternative mode of action of the Na pump, namely, the K activated phosphatase activity. Eosin also inhibited the pNPPase activity with an IC_{50} of 3.8 ± 0.23uM (Fig. 4A). Interestingly, it appears that eosin was unable to completely block this activity as maximal inhibition appears to plateau at about 80% (Fig. 4A).

Similar to Na,K-ATPase, when eosin inhibition was determined in the presence of increasing pNPP concentration the two compounds did not appear to be mutually exclusive (Fig. 4B). In constrast, when the purified enzyme was further treated with the nonionic detergent $C_{12}E_8$ (0.1%), the inhibition by eosin appears to become competitive with pNPP (Fig. 5). Increasing [pNPP] was able to displace eosin and the enzyme is able to attain the same maximal velocity (easily observed as all lines intersect the y-axis at the same point in the double-reciprocal plot shown in Fig. 5B). Na,K-ATPase treatment with $C_{12}E_8$ has been suggested to ensure that the enzyme is a single functional protomer (i.e. a single α/β complex) [30,31]. We also reexamined eosin and ATP competition for Na,K-ATPase on $C_{12}E_8$-treated enzyme, but even in this case eosin appeared to be a mixed-type rather than a competitive inhibitor (data not shown).

Discussion

Because eosin has been used as an ATP site probe in Na pump studies for the past 25 years, we expected that eosin would compete with ATP and pNPP during the Na,K-ATPase reaction and the K activated phosphatase activity. However, we found that eosin was a mixed-type inhibitor for both of these reactions. In general mixed inhibition implies that both substrate

(ATP or pNPP) and eosin can be bound to the pump, at least in one conformation and therefore that their binding is not mutually exclusive.

In this discussion we review the previous evidence in support of a model in which ATP and eosin bind in a mutually exclusive manner at the high affinity nucleotide site. We then discuss an unconventional model that stretches the mutually exclusive model to accommodate the mixed inhibition. Finally we discuss potential explanations for the change in kinetics observed in the presence of $C_{12}E_8$.

Previous work

Skou and Esmann [15] have shown that eosin and ATP compete for Na (only) ATPase consistent with their binding to the same site in a mutually exclusive manner. In contrast, we found that eosin was a mixed inhibitor. However the present study differs from Skou and Esmann's work in that here Na,K ATPase activity or K activated pNPPase was measured, whereas the earlier study focused on Na (only) ATPase activity. One obvious difference between these different enzymatic measurements is that during Na,K-ATPase or K activated pNPPase, there is a considerable contribution to k_{cat} by a low affinity ATP binding site that does not occur in the Na (only) ATPase measurement. Thus our results are not in conflict with those of Skou and Esmann, but by examining additional parts of the pump cycle, we have revealed a conformation that behaves differently than those involved in Na (only) ATPase.

Following the initial findings by Skou and Esmann that eosin competes with ATP for Na (only) ATPase activity [15], eosin has been used as a fluorescent probe to monitor conformational changes at the high affinity ATP site. Specifically, fluorescence fluctuations during ligand-induced conformational changes have been used to deduce coordinated movements between specific domains of P-type ATPases [16,18,19,33,34]. Interestingly, initial investigations did not observe any measurable eosin binding (as monitored by fluorescence changes) to the K^+-dependent portions of the catalytic cycle for either the Na,K-ATPase or H,K-ATPase suggesting that eosin was only a high affinity ATP binding site probe for P-type ATPases [15,16], but recent work suggests that eosin does bind to K^+ liganded conformations, but it cannot be easily detected by fluorescence changes [19]. Additional support for eosin binding to the ATP site comes from our observation that eosin protects Lys-501 from covalent modification by fluorescein isothiocyanate (FITC) (Fig. 3), which is not surprising since eosin is tetrabromofluorescein. Lysine-501 is a conserved residue among P-type ATPases that has been shown to coordinate ATP binding in high resolution structures of SERCA [14,37].

Eosin, in addition to inhibiting the Na pump, also inhibits the plasma membrane Ca pump from red cells [20] and smooth muscle [35]. Interestingly, those studies found that eosin was not competitive with ATP, similar to the results presented here for the Na pump. Because the Ca pump does Ca^{++}/nH^+ exchange, it is likely that those experiments were measuring analogous conformations to those observed during Na, K ATPase. Indeed, we are unaware of any attempts to measure a Ca ATPase activity analogous to Na only ATPase. Obviously, one cannot have $H^+ = 0$, and at very low H^+, there may be nonspecific affects on the Ca pump.

Earlier we suggested that the mixed type inhibition observed for red cell PMCA may be simply do the fact that eosin can bind the enzyme in E1 as well as E1P (see scheme 1), whereas ATP only binds to E1 with high affinity [28]. In this case, some of the amino acid contact residues for eosin remain the same, but the two enzyme conformations are kinetically distinct. One prediction of this model would be that eosin should compete with ADP for the dephosphorylation of ADP-sensitive EP as shown in Fig. 6A. This model is consistent with eosin acting as a mixed-type inhibitor even though it resides within the high affinity ATP site. However, this model would predict that eosin should be a mixed-type inhibitor of Na (only) ATPase and that was not observed [15], so this model does not apply to the Na pump.

Eosin Inhibition of K^+ activated Phosphatase Activity

In addition to ATP, the P-type pumps can hydrolyze other phosphate-containing substrates such as *para*-nitrophenyl phosphate [26,38,39,40], 3-*O*-methylfluorescein phosphate [41,42], and acetylphosphate [43,44]. We observed that eosin was a potent inhibitor of *p*NPPase activity (IC_{50} = 3.8 ± 0.23 μM; Fig. 4A). Indeed, this appears to be the first report directly measuring eosin inhibition of pNPPase activity in native Na pump, aside from one report showing that eosin blocks the subtle residual pNPPase activity associated with a bacterially expressed M4M5 loop from rat Na,K-ATPase [17].

Eosin inhibition of pNPPase may seem surprising, given that FITC-labeled enzyme remains capable of phosphatase activity (40, 41). However, previous work has also established that ATP still inhibits pNPPase in FITC treated Na pump. One explanation is that during the conformational change from E1 to E2, the tethered FITC molecule is swung out of the active site which allows pNPPase activity [41], but because eosin is not tethered, it is either bound at the site or not attached to the enzyme. An alternative model for the response of the FITC modified pump proposed by Martin and Sachs [40] is that an additional low affinity site for TNP-ADP and erythrosin isothiocyanate appears only after FITC-modification and likely represent a non-physiological allosteric inhibition site. This hypothesis is supported by the dramatically reduced affinities for both TNP-ADP and erythrosin isothiocyanate inhibition of the pNPPase activity in the FITC modified pump compared to the native enzyme [40]. Indeed, our finding that eosin inhibits pNPPase in the non-modified enzyme with high affinity is consistent with this hypothesis. However, Martin and Sachs suggest that pNPPase occurs at the catalytic site (and we tend to agree), yet we find that eosin inhibition of pNPPase is not competitive with pNPP in the native Na,K-ATPase (Fig. 4B).

Model of eosin inhibition of the Na pump

Mixed inhibition typically is explained with models in which the inhibitor binds to a separate site from the substrate. However, because of the substantial evidence that eosin binds to the ATP site, we sought to develop a model that encompasses all the key data and that has eosin and ATP sharing the same site. Specifically, the following key features of eosin inhibition of intact Na pump are included:

- **i.** eosin competes with ATP on Na (only) ATPase (15)
- **ii.** eosin is a mixed inhibitor with respect to ATP on Na,K-ATPase (Figures 1 and 2)
- **iii.** eosin is a mixed inhibitor with respect to pNPP for K activated phosphatase (Figure 5)
- **iv.** eosin protects the pump from reaction with FITC (Figure 3)
- **v.** eosin stimulates K^+ deocclusion and high eosin prevents the higher stimulation by ATP (18)
- **vi.** eosin does not completely inhibit pNPPase activity (Figure 4).

The simplest model that we can develop which is consistent with the data and has eosin and ATP sharing the same site, is a model in which this site is flexible. In certain conformations this site is able to bind both eosin and ATP (Fig. 6B). In other conformations, the site is unable to bind both, thus eosin and ATP are mutually exclusive (Fig. 6C.) It should be pointed out that Esmann and Fedosova [45] have shown that the site can simultaneously accommodate nitrate and eosin (and presumably nitrate and ADP) which supports the idea that at least sometimes, the site can stretch. In the presence of Na^+ and the absence of K^+, the conformation is such that eosin and ATP are mutually exclusive [45]. This is consistent with the earlier competition for Na (only)ATPase data, and the fact that ATP prevents the increase in eosin

fluorescence due to binding to the pump [15]. However, there exists one or more conformations during the Na, K ATPase cycle and during the pNPPase cycle in which the site can stretch and accommodate both eosin and ATP, thus explaining why high ATP or pNPP, respectively, cannot overcome the inhibition by eosin under these conditions.

A candidate for a conformation that allows the nucleotide site to stretch and accommodate both ATP (or pNPP) and eosin is the occluded state. However, the recent work by Rossi [18,19] seems to suggest that in the presence of K^+, eosin and ATP would be mutually exclusive as eosin prevents the extra stimulation of deocclusion when ATP is present. They found that while eosin can accelerate Rb^+ deocclusion, it was not as large an acceleration as observed with ATP [19]. At high eosin, ATP was not able to stimulate the rate, suggesting that eosin prevented ATP from binding. But an alternative explanation is that the site could stretch in this conformation and allow both ATP and eosin to bind, but that this stretched conformation cannot deocclude K^+ as fast as the "normal" conformation with ATP present. Thus, this conformation remains a candidate as one of the conformation(s) of the pump which allow the site to stretch and accommodate both eosin and ATP (or pNPP) to bind.

$C_{12}E_8$ treated pump

The Na pump behavior changes in the presence of $C_{12}E_8$: the addition of $C_{12}E_8$ changes the inhibition by eosin from mixed to competitive with pNPP. However a difficult constraint to the modeling is that eosin remains a mixed inhibitor for Na,K-ATPase.

$C_{12}E_8$ treatment shifts the equilibrium of pump from a diprotomer state to a monoprotomer state. $C_{12}E_8$ may also have additional effects. Clearly, all of the ion binding, transport, and enzymatic properties of the Na,K-ATPase can be catalyzed by a single functioning protomer consisting of one α and one β. Nonetheless, there continues to be discussions of and evidence for diprotomeric (or high oligoprotomeric) functioning Na,K-ATPase. Both camps have valid arguments. The single protomeric camp is justified in questioning the need to invoke oligomers when a single protomer can explain the vast majority of the existing data. However, since higher oligomers can clearly be isolated from membrane factions, the oligomeric camp is justified in questioning why oligmers exist if they are physiologically irrelevant. In an attempt to satisfy both camps we propose two possibilities for our *p*NPPase observations.

Diprotomer model—In this model, in the absence of $C_{12}E_8$, eosin binds to the catalytic pNPP binding site on one protomer, and the contacts with the second protomer allow both eosin and pNPP to fit within the pocket in one or more (but not all) conformations. This change in extensibility could reflect contacts between the P domain on one monomer and the N domain on the other as seen in the first high resolution SERCA crystal structure (14). Treatment with $C_{12}E_8$ separates the diprotomer to a predominate population of monoprotomers. Protomers are fully capable of hydrolyzing pNPP, but the site is not able to stretch to accommodate eosin and pNPP because the contact points on the other protomer are no longer close by. However, in a conformation involved in Na,K ATPase, but not pNPPase, even the monoprotomer is able to stretch and bind both ATP and eosin. While this model is appealing, we cannot explain why the stretch still occurs during Na,K-ATPase but does not during pNPPase.

Single protomer model—In this model, $C_{12}E_8$ causes a mild unfolding of the protein such that, while the enzyme can still stretch in $C_{12}E_8$, the rate of the rate limiting step for pNPPase activity is the same when eosin and pNPP are both bound or when just pNPP is bound. (The effect of eosin is merely to lower the affinity for pNPP, so that, at low pNPP eosin inhibits.) In contrast, in the native state, the pNPPase rate is slower with both bound to the stretched substrate site. One must also assume that the rate limiting step for Na,K-ATPase is slower when eosin and ATP are bound compared to just ATP bound, in both the native and $C_{12}E_8$

structure. Since the Na,K-ATPase cycle involves additional conformations compared to pNPPase, this is plausible.

Although neither model is completely satisfactory, they both emphasize that eosin inhibition of the Na pump appears to be more complicated than merely binding to the high affinity ATP site. Consequently, experiments which utilize eosin as a real-time fluorescent indicator of ATP site conformational changes are more safely interpreted if they include the possibility that ATP and eosin are not always mutually exclusive.

There are some potential tests to distinguish these models. The key difference between the models is whether the effect of $C_{12}E_8$ on the type of inhibition by eosin is due to the shift from diprotomer to monoprotomer or whether it is due to a slight unfolding of the pump. Since the Na pump in red blood cells is a monoprotomer of Na pump, if pNPP and eosin compete in the red cell, this would support the diprotomer model. On the other hand, if pNPP and eosin are mixed in the red cell, this would suggest that the shift in inhibition type by $C_{12}E_8$ on the kidney enzyme is due to it causing a slight unfolding. However, such data would not rule out the diprotomer model if band 3 associates with the monoprotomer and mimics the effect of the other Na pump in the diprotomer. This possibility could be tested in band 3 knockout animals which lack band 3 in their red cells, if the Na pump in these red cells remains in its monoprotomer state.

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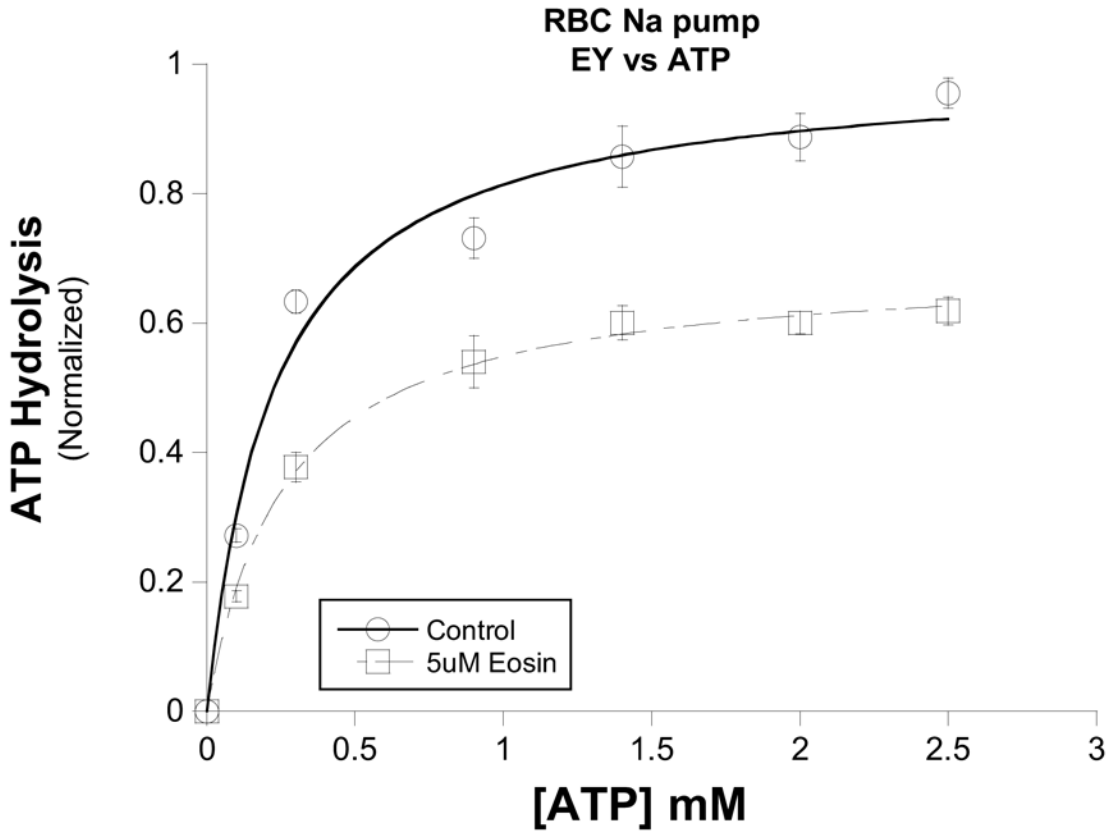
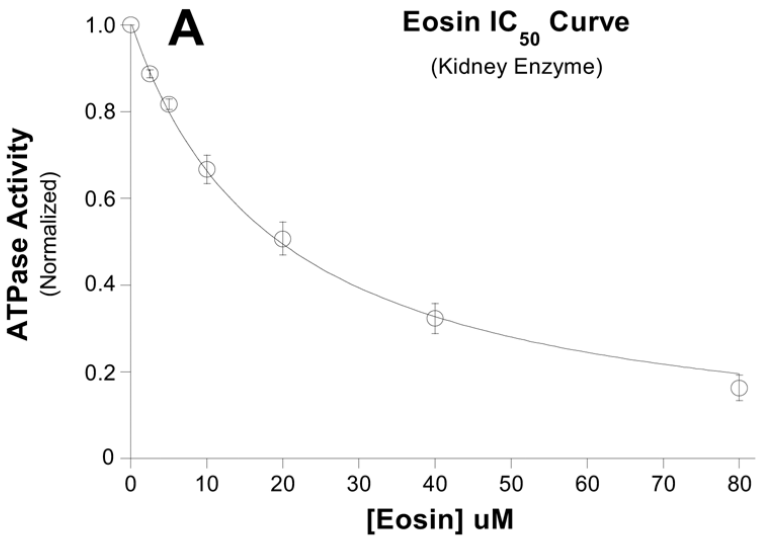


Figure 1. ATP-dependence of eosin inhibition of the pig red cell Na,K-ATPase

Ouabain-sensitive ATPase activity was measured in the presence of saturating substrates and the indicated amount of ATP. Increasing concentrations of ATP in the presence of 5μM eosin (□) were unable to displace eosin and attain control (○) V_{max} levels. Control V_{max}= 1.00 ± 0.04, $K_{1/2}$ = 0.23 ± 0.04 mM; Eosin V_{max} = 0.69 ± 0.01, $K_{1/2}$ = 0.26 ± 0.02 mM. Data from three different experiments were normalized to maximal velocity of ATP hydrolysis in the absence of inhibitor. Points represent means ± SEM.



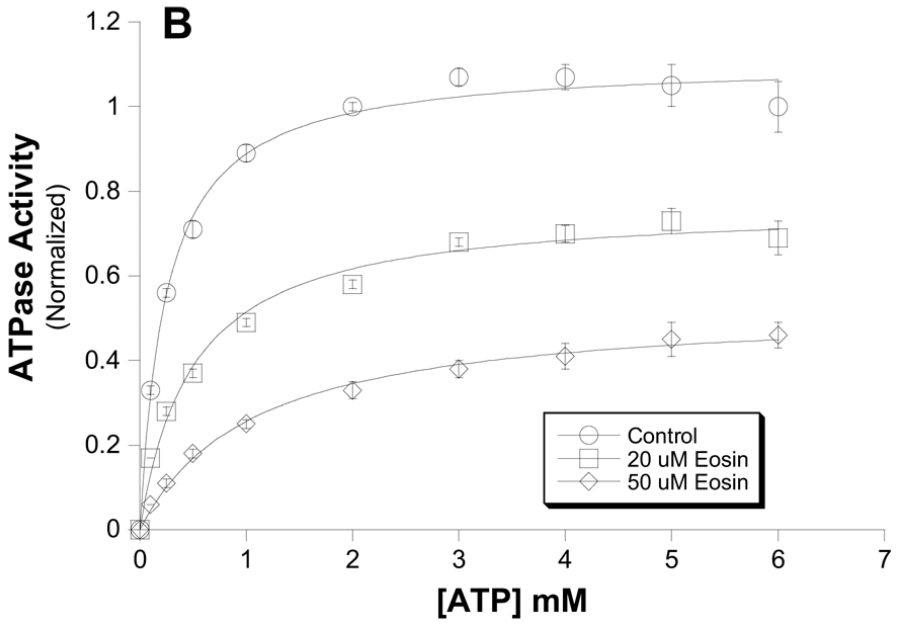


Figure 2. Eosin inhibition of purified renal Na,K-ATPase

Ouabain-sensitive ATPase activity was measured in the presence of saturating substrates and the indicated amount of ATP. A.) ATP-dependence of eosin inhibition of kidney Na,K-ATPase. Similar to the red cell Na pump (Fig. 1), increasing concentrations of ATP in the presence of either 20μM (□) or 50μM eosin (○) were unable to displace eosin and attain control V_{max} levels. Control V_{max}= 1.11 ± 0.02, $K_{1/2}$ = 0.25 ± 0.02mM; 20uM Eosin V_{max} = 0.77 ± 0.02, $K_{1/2}$ = 0.49 ± 0.06mM; 50uM Eosin V_{max} = 0.53 ± 0.01, $K_{1/2}$ = 1.0 ± 0.10mM. Data from three different experiments were normalized to maximal velocity of ATP hydrolysis in the absence of inhibitor. Points represent means ± SEM. B.) Dose-dependence of eosin inhibition on Na,K-ATPase. The inhibitory effect of eosin was measured on ouabain-sensitive Na,K-ATPase from

purified sheep kidney enzyme. The data were fit to the equation $v = V * IC_{50}/ (IC_{50}+I)$ where V is the maximal velocity in the absence of eosin (i.e. I=0), and I is [eosin]. The IC_{50} was 19.2 ± 1.01μM. Data were normalized to the activity in the absence of eosin.

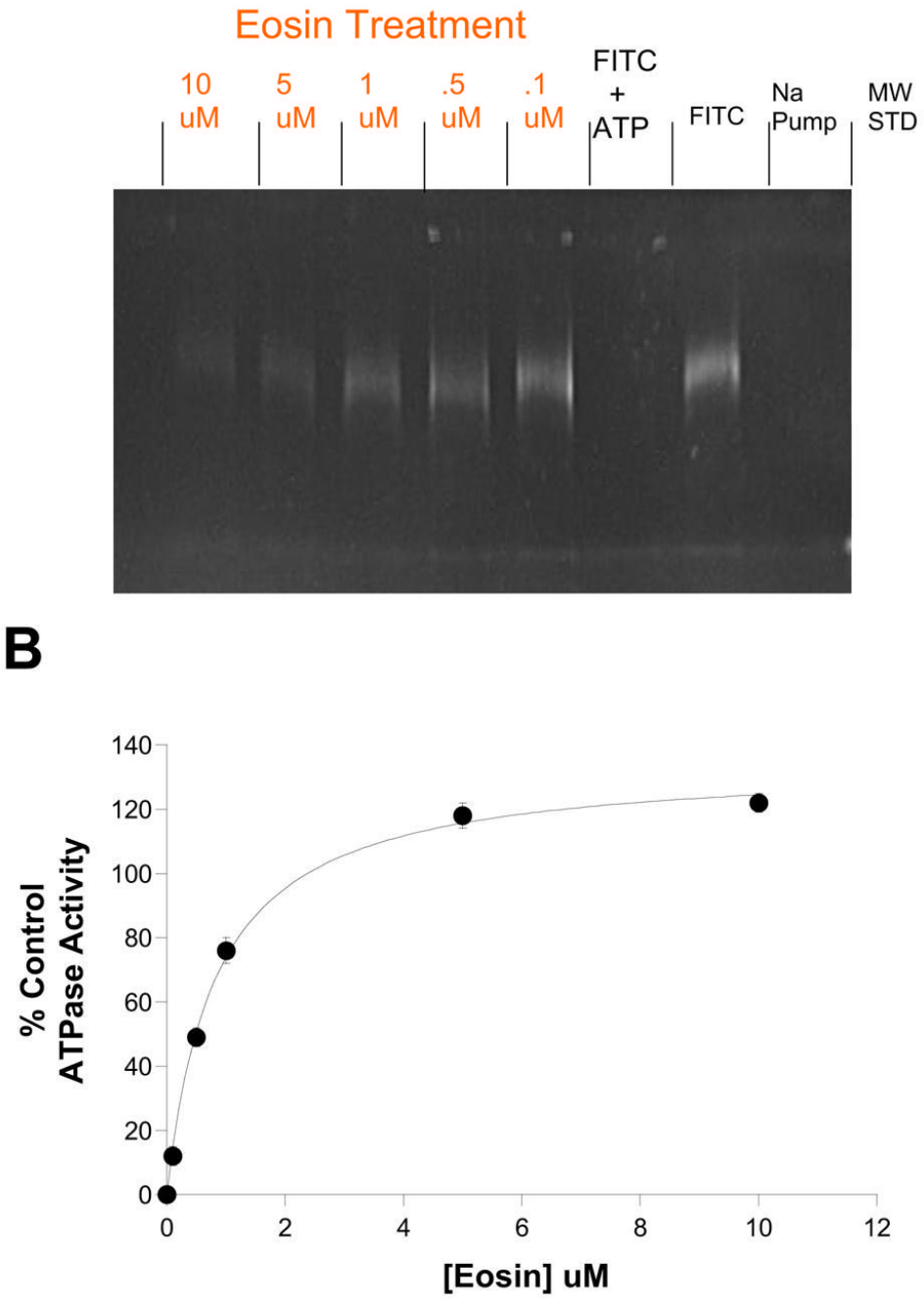


Figure 3. Eosin Protects against covalent modification by FITC

A.) FITC covalently labels Lys-501 of the Na,K-ATPase nucleotide binding domain of Na,K-ATPase. The FITC-modified alpha subunit can be detected by UV illumination on SDS-PAGE (FITC lane). Moreover, the simultaneous presence of 3mM ATP protects against FITC (FITC +ATP lane). FITC labeling was inhibited by eosin in a dose-dependent fashion (Eosin Treatment lanes, [eosin] range = 0.1 – 10 μM as indicated). **B.)** Eosin dose-response curve for protection against FITC-inactivation of Na,K-ATPase Activity. Aliquots from the FITC-labeled enzyme shown in **A** were diluted 120-fold and ouabain-sensitive ATPase activity measured and compared to the non-FITC modified purified sheep renal Na,K-ATPase. The

$K_{1/2}$ for eosin protection was 0.82 ±0.6μM. Data points are means and bars are standard deviation of a single experiment, representative of two separate experiments.

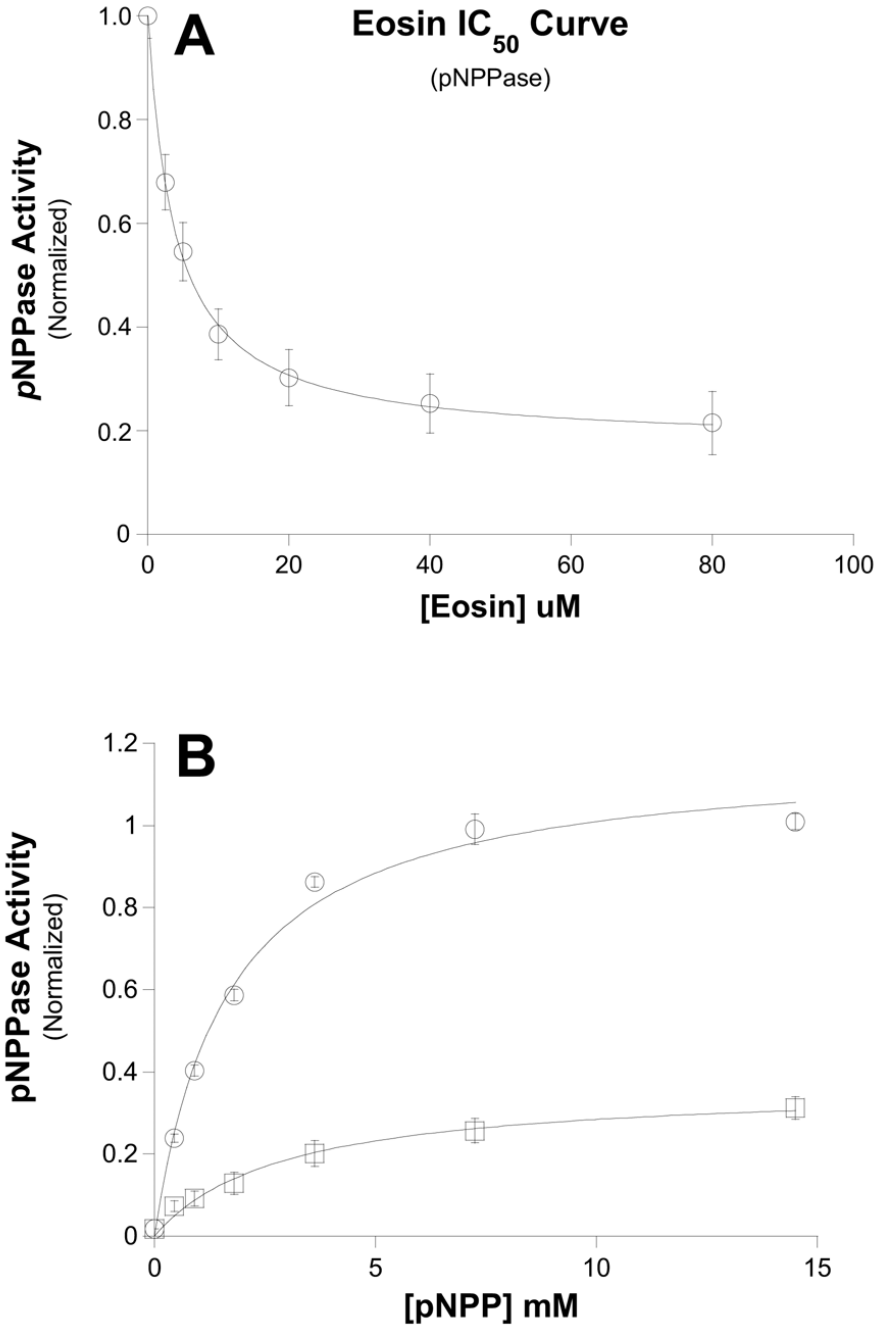


Figure 4. Dose-dependence of eosin inhibition on K^+-dependent phosphatase activity

Ouabain-sensitive, K^+-dependent *p*NPPase activity was measured in the presence of the indicated concentrations of eosin (see Methods). Data were fit to the same equation show in Fig. 1. Interestingly, the IC_{50} curve appears to plateau with a residual noneosin-sensitive 20% activity. The IC_{50} for eosin-sensitive component was 3.8 ± 0.23 μM. Data points are means and bars are standard error of six separate experiments. **B.)** *p*NPP dose-dependence of eosin inhibition. K^+-dependent phosphatase activity was measured with increasing [pNPP] in the absence (○) and presence (□) of 25μM eosin. Data were fit to the Michaelis-Menten equation. Control V_{max} = 1.17 ± 0.05; $K_{1/2}$ = 1.65 ± 0.21 μM; 25 μM eosin V_{max} = 0.38 ± 0.05; $K_{1/2}$ =

2.91 ± 0.54 μM. These data suggest that eosin does not compete with pNPP. Data points are means and bars are standard error of three separate experiments.

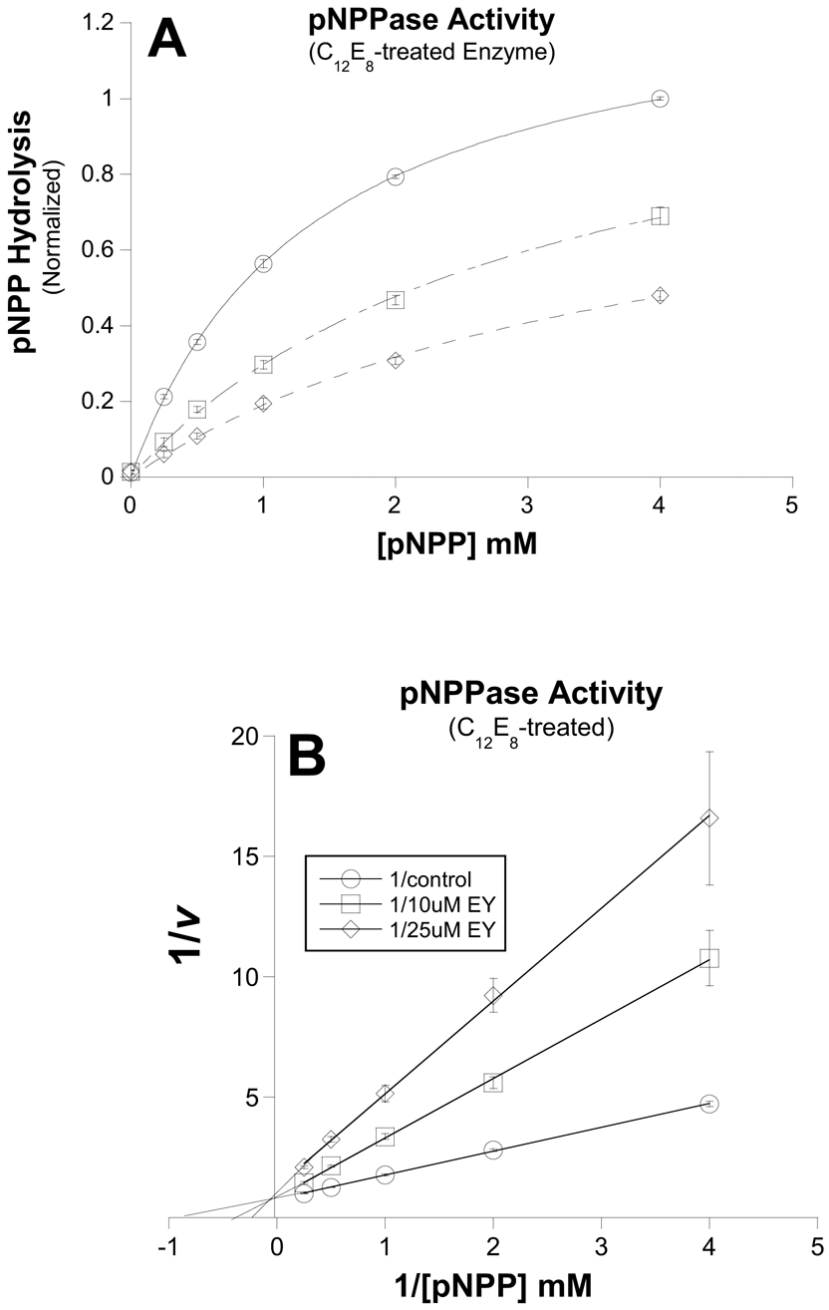


Figure 5. pNPP concentration-dependence of eosin inhibition of K^+-dependent Phosphatase activity

pNPP-activation of phosphatase activity was measured in the absence (○) or presence of 10uM (□) or 25uM (◇) eosin. **A.)** Michaelis-Menten plot of data. In contrast to its effects on the phosphatase reaction of untreated enzyme, eosin appears to compete with pNPP for C12E8-treated enzyme. Control V_{max} = 1.3 ± 0.02; $K_{1/2}$ = 1.4 ± 0.04 μM; 10 μM eosin V_{max} = 1.2 ± 0.06; $K_{1/2}$ = 3.1 ± 0.26 μM; 25 μM eosin V_{max} = 1.0 ± 0.07; $K_{1/2}$ = 4.0 ± 0.51 μM. **B.)** Double-Reciprocal plot of the data shown in fig. A. Here it is clear that the three lines intersect at the y-axis, consistent with eosin and pNPP binding in a mutually exclusive manner. Data points are means and bars are standard error of three separate experiments.

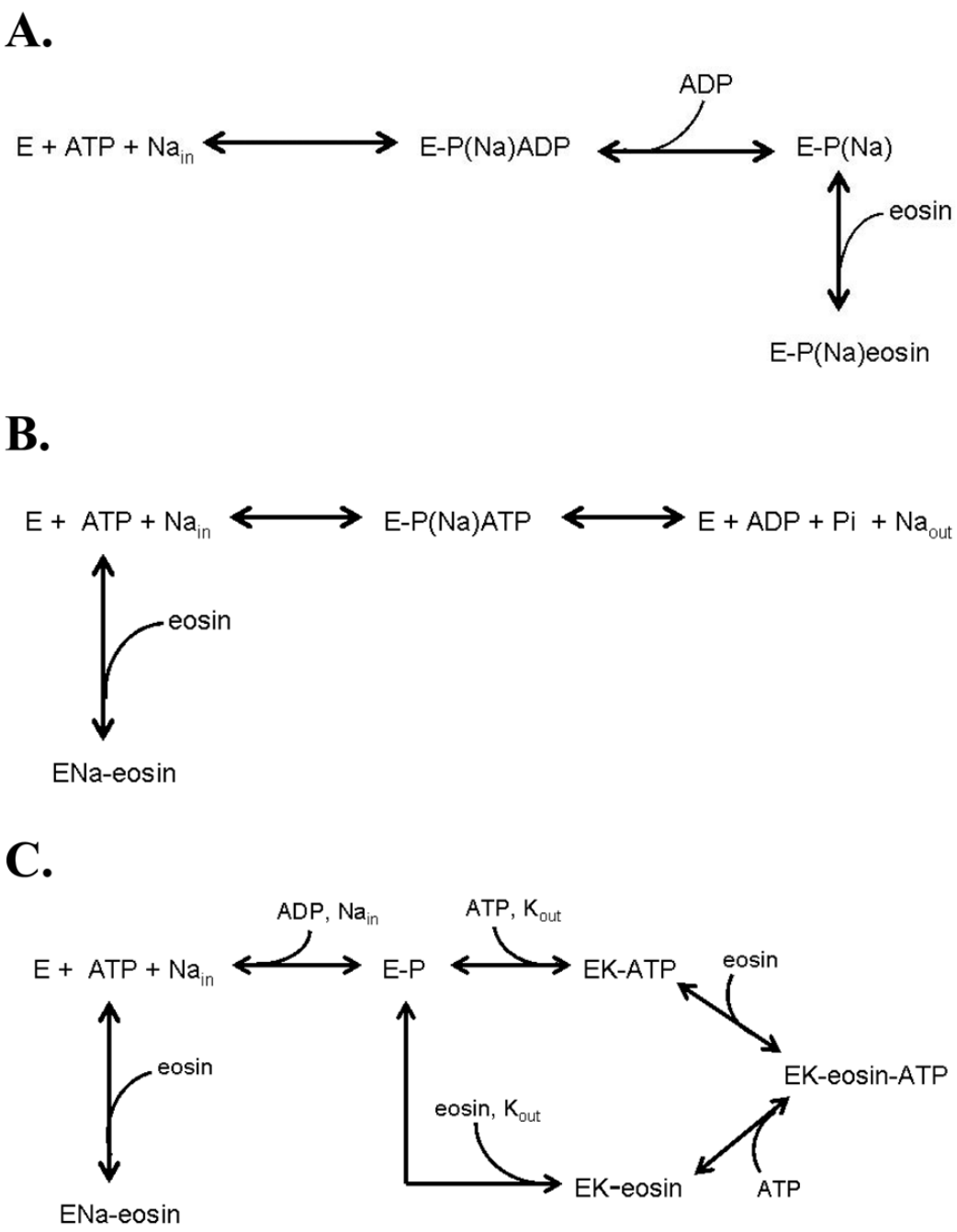


Figure 6. Potential kinetic schemes for eosin inhibition of the Na pump

A. Eosin and ADP compete which is consistent with eosin being a mixed inhibitor of ATP for Na,K ATPase. It is not consistent with eosin being a competitive inhibitor of Na (only) ATPase.

B. Eosin and ATP compete for Na (only) ATPase.

C. Eosin and ATP can both bind for Na,K ATPase.