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SODIUM AND POTASSIUM INTERACTIONS WITH Na^+-ATPase OF INSIDE-OUT MEMBRANE VESICLES FROM HIGH-K^+ AND LOW-K^+ SHEEP RED CELLS

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Summary

Na^+-ATPase of high-K^+ and low-K^+ sheep red cells was examined with respect to the sidedness of Na^+ and K^+ effects, using inside-out membrane vesicles and very low ATP concentrations ($\leqslant 2$ μM). With varying amounts of Na^+ in the medium, i.e., at the cytoplasmic surface, Na^+_{cyt}, the activation curves show that high-K^+ Na^+-ATPase has a higher affinity for Na^+_{cyt} compared to low-K^+. The apparent affinity for Na^+_{cyt} is also increased by increasing the ATP concentrations in high-K^+ but not low-K^+. With Na^+_{cyt} present, Na^+-ATPase is stimulated by intravesicular Na^+, i.e., Na^+ at the originally external surface, Na^+_{ext}, to a greater extent in low-K^+ than high-K^+. Intravesicular K^+ (K^+_{ext}) activates Na^+-ATPase in high-K^+ but not in low-K^+ vesicles and extravesicular K^+ (K^+_{cyt}) inhibits low-K^+ but not high-K^+ Na^+-ATPase. Thus, the genetic difference between high-K^+ and low-K^+ is expressed as differences in apparent affinities for both Na^+ and K^+ and these differences are evident at both cytoplasmic and external membrane surfaces.

Introduction

Sheep are genetically dimorphic with respect to the intracellular K^+ content of their red cells. The cells of one type have high-K^+, the cells of the other type have low-K^+ concentrations, the latter being dominant [1]. Although the difference in the K^+ gradient across the red cell membrane is partly associated with differences in the rate of ouabain-sensitive Na^+ and K^+ transport [2], ($Na^+ + K^+$)-ATPase activity [3] and number of ouabain binding sites [4—6],

different kinetic characteristics between high-K^+ and low-K^+ are also evident. Thus high-K^+ and low-K^+ pump sites differ in their affinities for intracellular K^+, low-K^+ being strongly inhibited by K^+ [7], and differ in their affinity for ouabain [8]. These differences are reflected also in studies of Na^+-ATPase assayed at very low concentrations of ATP under which condition the activity of low-K^+ is inhibited whereas the activity of high-K^+ is activated by K^+ ($KCl \leqslant 5$ mM) [9].

The present study was designed to test more directly the side-specificity of these K^+ effects on Na^+-ATPase by using inside-out membrane vesicles of the two types of sheep red cell. These vesicles are prepared according to the method described by Steck et al. [10] for human red cell membrane vesicles. This system allows not only the composition of the medium at the two surfaces to be precisely controlled, but also permits direct accessibility of the catalytic sites to the reaction medium. These advantages have proven useful in studies of alkali cation interactions with the sodium pump system of human red cells [11—13]. The results show that low-K^+ and high-K^+ Na^+-ATPase activities differ with respect to their interactions with Na^+ as well as K^+ and these differences are evident at both cytoplasmic and external membrane surfaces.

Materials and Methods

The vesicle preparation and Na^+-ATPase assays were carried out as described previously [11]. Neuraminidase-sensitive membrane sialic acid groups were measured as described by Perrone and Blostein [14]. Vesicles were loaded with cations as described elsewhere [11]. Overnight loading resulted in approx. 70% equilibration of ions (Na^+ or K^+) in high-K^+ and over 90% in low-K^+ vesicles. Protein was estimated by the method of Lowry et al. [15].

[γ-^{32}P]ATP was prepared as described previously [16]. The specific radioactivity was 5—10 Ci/mmol. Neuraminidase and valinomycin were purchased from Sigma Chemical Company. Each value is the average of duplicate or triplicate determinations and, unless indicated otherwise, results reported are representative of replicate (at least three) independent experiments.

Results

Sidedness of the vesicles

Since the vesicles used in these studies are inside-out, extravesicular Na^+ or K^+ is equivalent to Na^+ or K^+ at the cytoplasmic surface and is designated Na^+_{cyt} or K^+_{cyt}, respectively. Similarly, intravesicular Na^+ or K^+ is Na^+ or K^+ at the normally external surface and is designated Na^+_{ext} or K^+_{ext}, respectively.

The sealing and orientation of the sheep red cell vesicles were assessed as follows. The sensitivity of the Na^+-ATPase activity to ouabain inhibition was assayed since sealed inside-out vesicles should have their ouabain binding sites, originally at the external surface of the membrane, at the inner vesicular surface and thus inaccessible to ouabain binding and inhibition [14]. As can be seen from the data in Table I, both high-K^+ and low-K^+ Na^+-ATPase are about 90% insensitive to ouabain ($2 \cdot 10^{-5}$ M).

The susceptibility of sialic acid groups, present only at the originally external

TABLE I

EFFECT OF OUABAIN ON HIGH-K^+ AND LOW-K^+ Na^+-ATPase

Vesicles (approx. 1 mg/ml) were concentrated to one-tenth or one-fifth of their original volume and were added to 9 or 19 vols. of a reaction medium, respectively. The final volume, 0.1 ml, contained 20 mM NaCl, 10 mM Tris-glycylglycine (pH 7.4), and 0.1 mM $MgCl_2$. 0.02 mM ouabain was added as indicated. Assays of Na^+-ATPase of high-K^+ vesicles were carried out for 2 min at 37°C. The reaction was terminated and $^{32}P_i$ released from [γ-^{32}P]ATP was measured. Values are corrected for the activity measured in the absence of NaCl and presence of 20 mM KCl. In the high-K^+ assay indicated by an asterisk, the vesicles were exposed to 0.02 mM ouabain present both in the assay and during the vesiculation period, i.e., ouabain was present at the originally external surface, and an activity of 0.35 pmol · min^{-1} · mg^{-1} was observed. S.E. is the standard error of the mean.

Sheep type	ATP concentration	Na^+-ATPase (pmol · min^{-1} · mg^{-1})		
		Control	+0.02 mM ouabain	% control
High-K^+	0.2 μM	74.1	79.1	107
		45.8	37.6	82
		51.3	44.6	87
		40.6	34.8	86
		40.5	37.2 0.35 *	92
Mean ± S.E.		50.5 ± 7.5	46.7 ± 10	91 ± 5
Low-K^+	0.2 μM	2.8	2.5	89
		3.3	3.0	91
		1.4	1.0	72
		2.4	2.5	104
		1.4	1.2	86
Mean ± S.E.		2.3 ± 0.4	2.0 ± 0.4	88 ± 6

membrane surface [17], to cleavage by neuraminidase was assayed and the data are presented in Table II. Only 10% of the total sialic acid content of both high-K^+ and low-K^+ vesicles was removed by neuraminidase in the reaction medium, suggesting that the vesicles are about 90% inside-out.

Effects of Na^+

In Fig. 1 Na^+-ATPase activity was assayed at 0.02 and 0.2 μM ATP using varying amounts of Na^+_{cyt} (up to 40 mM). The 'control' values in Fig. 1 are the

TABLE II

SIALIC ACID DISTRIBUTION IN HIGH-K^+ AND LOW-K^+ INSIDE-OUT VESICLES

For the measurement of total sialic acid, vesicles were diluted 2.5-fold and were heated at 80°C for 1 h in a final volume of 0.25 ml containing 0.2 N H_2SO_4. Neuraminidase-sensitive sialic acid was measured following incubation at 37°C for 1 h in a final volume of 0.25 ml containing 0.2 M Tris-acetate (pH 5.7) and 5 μg neuraminidase.

Sheep type	Sialic acid content (nmol · mg^{-1})		
	Total	Neuraminidase-sensitive	% insensitive
High-K^+	90.7	9.0	90
	49.5	10.1	80
Low-K^+	125.3	8.0	94
	135.9	27.1	80
	46.5	5.9	87

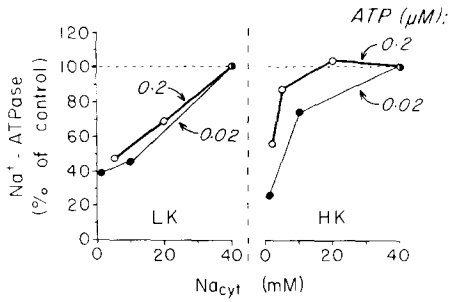

Fig. 1. Effect of Na^+_{cyt} on high-K^+ and low-K^+ Na^+-ATPase. Vesicles were concentrated to one-tenth their original volume by centrifugation and were added to 9 vols. of 45 mM choline chloride and 0.1 mM $MgCl_2$, centrifuged again and then allowed to equilibrate at 0°C overnight. For Na^+-ATPase assays, 1 vol. of vesicles was added to 9 vols. of a reaction medium in a final volume of 0.1 ml containing varying amounts of NaCl as indicated plus choline chloride so that the final chloride concentration was 40 mM, 10 mM Tris-glycylglycine (pH 7.4), 0.1 mM $MgCl_2$ and 0.02 or 0.2 μM [γ-^{32}P]ATP at 37°C for 2—5 min. The reaction was terminated and $^{32}P_i$ released was measured. The values are corrected for activity measured in the absence of NaCl and presence of 40 mM KCl. The values are expressed as the percentage of activity observed at 40 mM Na^+_{cyt} (control). LK, low-K^+; HK, high-K^+.

activities observed at the highest Na^+_{cyt} concentration tested. Baseline activities were measured without Na^+ and with K^+ added to counteract effects of any residual and/or tightly bound Na^+; these baseline values have been subtracted. At these low ATP concentrations, Na^+_{cyt} alone, even at 1 mM, significantly increases activity.

The response of the low-K^+ enzyme to Na^+_{cyt} is different from that of high-K^+ over the range of Na^+_{cyt} concentrations tested, the apparent affinity being clearly lower for low-K^+. This was apparent at both ATP concentrations tested. The considerable level of activity observed in the absence of added Na^+ (~40% of the activity observed at 40 mM Na^+_{cyt}) is likely due to traces of tightly bound Na^+.

Na^+-ATPase activity of high-K^+ vesicles tends to reach maximal activity above about 5 mM Na^+_{cyt}, particularly at 0.2 μM ATP. Increasing the ATP concentration decreased the level of Na^+_{cyt} required for maximal activation. With 40 mM Na^+_{cyt}, intravesicular Na^+ (Na^+ at the originally external surface, Na^+_{ext}) further increases the activity (Table III). The stimulation of activity by Na^+_{ext} is more marked in low-K^+ compared to high-K^+ vesicles.

Effects of K^+

Na^+-ATPase activity was assayed as a function of KCl in the media (K^+ at the originally cytoplasmic surface, K^+_{cyt}) or as a function of intravesicular K^+ (K^+_{ext}). Activities were assayed at 0.02, 0.2 and 2.0 μM ATP. As shown in Fig. 2, the activity of low-K^+ vesicles is inhibited by K^+ at either surface at all ATP concentrations tested, although K^+_{cyt} inhibition is always greater. In the experiment at 0.2 μM ATP (Fig. 2b), 1 μM valinomycin was included with the KCl in the reaction medium so that K^+ had access to both surfaces of the membrane. Addition of valinomycin does not increase the extent of inhibition by K^+ beyond that observed with K^+_{cyt} alone. Thus K^+_{cyt} appears to be responsible for inhibition of the enzyme. The data suggest also that the apparent K^+_{ext} inhibi-

TABLE III

EFFECT OF Na^+_{ext} ON HIGH-K^+ AND LOW-K^+ Na^+-ATPase

Vesicles were first concentrated to one-tenth their original volume by centrifugation and then diluted 19-fold with 52.5 mM choline chloride or NaCl containing 0.1 mM $MgCl_2$. They were then centrifuged again and allowed to equilibrate at 0°C overnight. For Na^+-ATPase measurements, 1 vol. of vesicles was then added to 9 vols. of a reaction medium in a final volume of 0.1 ml containing 40 mM NaCl, 10 mM Tris-glycylglycine (pH 7.4), 0.1 mM $MgCl_2$, and 0.02 or 0.2 μM [γ-^{32}P]ATP. The reaction was carried out for 2—5 min at 37°C and then terminated. The values are corrected for activity measured in the absence of NaCl and presence of 40 mM KCl. The values shown are the percentage of activity measured in Na^+-loaded vesicles relative to choline-loaded vesicles. S.D. is the standard deviation, *N* is the number of experiments performed, and *P* is the level of significance of Student's *t*-test performed on paired data, i.e., with and without Na^+_{ext}.

Sheep type	ATP concentration (μM)	% activity (mean ± S.D.)	*N*	*P*
High-K^+	0.02	105 ± 5	6	$0.1 < P < 0.05$
	0.20	124 ± 21	6	$0.05 < P < 0.01$
Low-K^+	0.02	124 ± 12	10	$P < 0.001$
	0.20	165 ± 40	11	$P < 0.001$

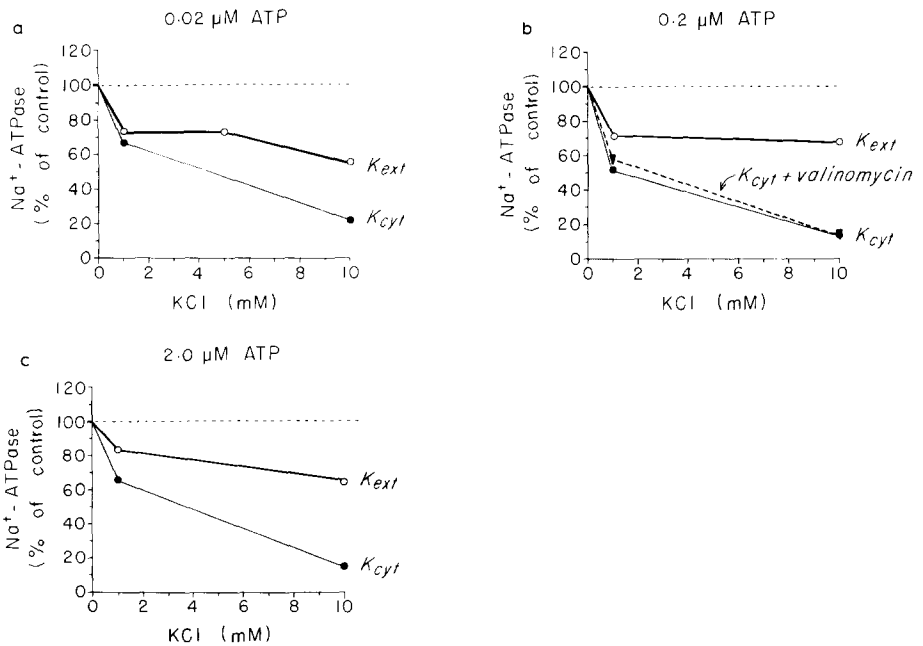

Fig. 2. Effect of K^+_{cyt} and K^+_{ext} on low-K^+ Na^+-ATPase. Vesicles were concentrated to one-tenth their original volume by centrifugation and were added to 9 vols. of a choline chloride/KCl solution so that the final KCl varied as indicated. The final chloride concentration was 40 mM. 0.1 mM $MgCl_2$ was present throughout. The vesicles were recentrifuged and allowed to equilibrate at 0° C overnight. 1 vol. of vesicles was added to 9 vols. of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 40 mM, 10 mM Tris-glycylglycine (pH 7.4), and 0.1 mM $MgCl_2$. After 5 min at 0.02 μM (Fig. 2a) and 0.2 μM (Fig. 2b) and 10 min at 2.0 μM [γ-^{32}P]ATP (Fig. 2c) at 37° C, the reaction was terminated and $^{32}P_i$ released was measured. The values are corrected for activity measured in the absence of NaCl and presence of 50 mM KCl. As indicated in Fig. 2b, 1 μM valinomycin was added (2.2 μl 0.45 mM valinomycin dissolved in absolute ethanol per ml reaction medium). The values are expressed as the percentage of activity observed in the absence of KCl (control).

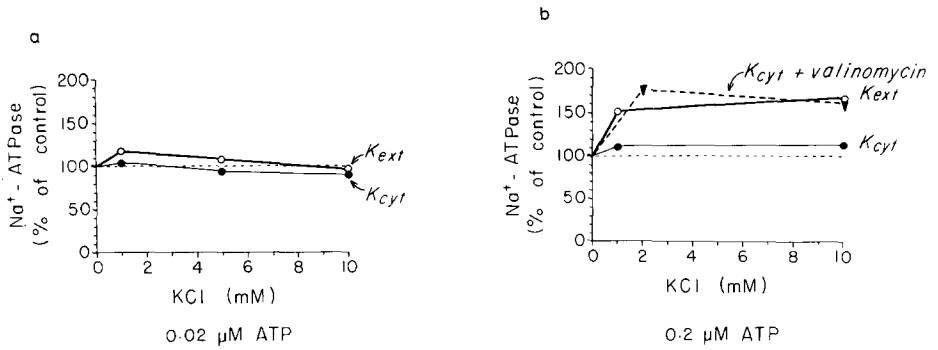

Fig. 3. Effect of K^+_{cyt} and K^+_{ext} on high-K^+ Na^+-ATPase. Vesicles were loaded and the reactions were carried out as described in Fig. 2. The reactions were performed for 5 min at 37°C at 0.02 μM (Fig. 3a) and 0.2 μM [γ-^{32}P]ATP (Fig. 3b). As indicated in Fig. 3b, 1 μM valinomycin was added as described in Fig. 2. The values are expressed as the percentage of activity observed in the absence of KCl (control).

tion is due to K^+_{cyt} present since 10-fold dilution of K^+-equilibrated vesicles results in K^+_{cyt} present at one-tenth the K^+_{ext} concentration.

With 0.02 μM ATP, Na^+-ATPase of high-K^+ vesicles shows relatively little response to addition of K^+ at either surface (Fig. 3a). However, at 0.2 μM ATP, K^+_{ext} markedly activates the Na^+-ATPase and K^+_{cyt} effects only a slight activation (Fig. 3b), probably because of slight K^+ leaking to the 'external' surface. At 2 μM ATP (data not shown), KCl increased activity several-fold, characteristic of the Na^+- plus K^+-dependent activation of the ATPase observed at high ATP concentrations.

Discussion

The results of this study show several differences at both membrane surfaces of Na^+ and K^+ interactions with (Na^+ + K^+)-ATPase of high-K^+ and low-K^+ red cells. Most earlier studies (see Refs. 9 and 18) were carried out with fragmented membranes devoid of the asymmetry required to define the surfaces at which the cations were acting, although in one study with goat low-K^+ cells [19], appropriate corrections were introduced to account for effects of K^+_{ext}.

From a comparison of the effects of Na^+ and K^+ on Na^+-ATPase of both types of vesicle, the following are evident:

(i) K^+ stimulates high-K^+ and inhibits low-K^+ Na^+-ATPase, these effects occurring at the external surface of high-K^+ and cytoplasmic surface of low-K^+ red cell vesicles. The activation of the high-K^+ Na^+-ATPase by K^+_{ext} is almost maximal with 1 mM K^+ and is, presumably, at the high-affinity K^+ loading site [20,21]. The strong inhibition by K^+_{cyt} in low-K^+ vesicles is most likely due to competition for Na^+ at the Na^+ loading site as in the case of human red cell (Na^+ + K^+)-ATPase [11]. These observations agree also with the earlier reports of inhibition by intracellular K^+ in both low-K^+ sheep [7,22—24] and goat [19] red cells.

(ii) Vesicles of high-K^+ cells have a greater apparent affinity for Na^+_{cyt} and are activated to a greater extent than low-K^+. The response to Na^+_{cyt} is similar to

the observed effects of Na^+ on ATPase of broken membranes from the two types of sheep red cells [18]. Whereas increasing the ATP concentration (from 0.02 to 0.2 μM) increased the apparent affinity of high-K^+ Na^+-ATPase for Na^+_{cyt}, this did not affect the response of the low-K^+ enzyme to Na^+_{cyt}. Thus ATP, even at these low concentrations, is observed to modulate the affinity of the high-K^+ enzyme for Na^+_{cyt}, presumably at its loading site, whereas the low-K^+ enzyme is less sensitive to changes in concentration of both Na^+_{cyt} and ATP.

(iii) In the absence of K^+, Na^+_{ext} appears to have a further activating effect on the enzyme, to a greater extent in low-K^+ than high-K^+ (see Table III). With human Na^+-ATPase, this Na^+_{ext} stimulation observed at very low ATP concentrations has been accounted for in terms of current models for the reaction sequence [13]. Thus, in accordance with the scheme shown in Fig. 4, a step in the transition of phosphoenzyme from one form, E_1-P, to another, E_2-P, is reversed by Na^+_{ext} with the result that hydrolysis proceeds mainly by an alternate route (E_1-P $\rightarrow$ E_1 + P_i). This alternate route [25] may result in a greater overall rate of ATP hydrolysis if subsequent regeneration of the active E_1 form of the enzyme from E_2 via the normal route is dependent on a modulating effect of ATP.

It is now clear that the higher rates of Na^+ and K^+ transport [2], (Na^+ + K^+)-ATPase [3] and Na^+-ATPase [9] activities in high-K^+ compared to low-K^+ red cells are due not only to a somewhat greater number of pump sites per cell [4—6,9] but also due to kinetic differences. These differences can be considered in terms of the plausible reaction steps involving side-specific interactions of Na^+ and K^+ at the two membrane surfaces. In Fig. 4, E_1 refers to a Na^+-sensitive form of the enzyme, the form in which the phosphoryl transfer reaction from ATP occurs, and E_2 refers to a K^+-sensitive form [26—28]. The clockwise (normal) cycle of reactions involves first a Na^+_{cyt}-dependent phosphorylation of E_1 to form a phosphorylated form E_1-P [13] followed by a transition of E_1-P to E_2-P [29]. This is then followed by K^+_{ext}-stimulated dephosphorylation of E_2-P [13] and reversion of E_2 back to E_1.

Assuming that K^+ binding at the external surface results in P_i release from phosphoenzyme, i.e., E_2-P + K^+_{ext} $\rightarrow$ (K^+)E_2 + P_i, it is possible that the rate of release of K^+ from (K^+)E_2 may determine whether the enzyme is activated by

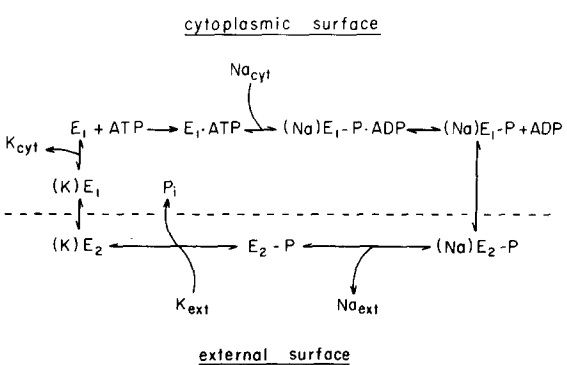

Fig. 4. Reaction scheme for (Na^+ + K^+)-ATPase.

K^+_{ext}. As mentioned above, several studies [30—33] suggest that ATP has a role in regulating the release of K^+ from the enzyme and/or the conversion of E_2 back to E_1. It is apparent that at low ATP concentrations the enzyme is stimulated maximally by Na^+; at higher ATP concentrations, maximal stimulation is observed with both Na^+ plus K^+. The interaction of ATP with the enzyme at the catalytic (phosphorylation) site and at a regulatory site may be interrelated. This would be the case, for example, if ($Na^+ + K^+$)-ATPase were a dimeric enzyme having half-the-sites reactivity as suggested by others [34].

A basic kinetic difference between high-K^+ and low-K^+ Na^+-ATPase may be their interaction with ATP which is manifested by different relative affinities of the dephosphoenzyme for Na^+_{cyt} and K^+_{cyt} as well as effects of Na^+_{ext} and K^+_{ext} on the phosphoenzyme. Thus, if the regulatory effect of ATP is on release of K^+ from the enzyme, a weak interaction of the low-K^+ enzyme with ATP could not only make this step rate-limiting and insensitive to K^+_{ext}-stimulation of dephosphorylation (E_2-P + $K^+_{ext} \rightarrow (K^+)E_2 + P_i$), but could also explain the observation that K^+ inhibits the enzyme at both surfaces. The effects of K^+ at both surfaces are consistent with our recent study [12] on the sidedness of K^+ activation of *p*-nitrophenylphosphatase of human red blood cell membrane vesicles [12]. The results are consistent with a model whereby a K^+-form of the 'dephospho' enzyme, probably $(K^+)E_2$, catalyzes the phosphatase reaction; formation of $(K^+)E_2$ can occur either via K^+_{cyt} interaction with the dephosphoenzyme or via K^+_{ext} interaction with the phosphoenzyme, in accord with the scheme in Fig. 4. ATP counteracts the former but promotes the latter reaction. Thus it is not surprising that tight binding of K^+ to the dephosphoenzyme is evident in differences between high-K^+ and low-K^+ Na^+-ATPase with respect to interactions with ligands at both membrane surfaces.

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