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# Cation sites, spermine, and the reaction sequence of the $(Na^+ + K^+)$-dependent ATPase

Joseph D. Robinson *, Catherine A. Leach and Lisa J. Robinson

*Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, NY 13210 (U.S.A.)*

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**Spermine, at 0.3 mM, inhibits the $K^+$-nitrophenyl phosphatase activity of a dog kidney $(Na^+ + K^+)$-ATPase preparation, increasing the $K_{0.5}$ for $K^+$, reducing the $K_m$ for substrate, and affecting little the inhibition by $Na^+$. These actions can be attributed, in a model of the phosphatase reaction, to parallel decreases in affinity for $K^+$ and $Na^+$ at their cytoplasmically accessible sites. In the $(Na^+ + K^+)$-ATPase reaction, spermine increases the $K_{0.5}$ for $Na^+$ and, to a lesser degree, the $K_{0.5}$ for $K^+$ as activators. With spermine, the double-reciprocal plots of velocity vs. ATP concentration (in the range 0.3–3 mM), at fixed levels of $K^+$ (from 1 to 10 mM), remain parallel but are rotated clockwise and spread somewhat, reflecting stimulation at low ATP concentrations and inhibition at high ATP but low KCl concentrations. These actions can be attributed, in a steady-state ping-pong model of the ATPase reaction, solely to decreased rates of binding of $Na^+$ and $K^+$ to their sites, with major effects at the cytoplasmically accessible sites for $Na^+$ (acceptance) and $K^+$ (discharge), and with a lesser effect at the extracellularly accessible sites for $K^+$ (acceptance). On these grounds, spermine is a highly specific and potentially valuable reagent for studying the reaction. Furthermore, the model for $K^+$-ATP interactions not only supports a specific reaction sequence ($K^+$ addition, $P_i$ release, ATP addition, $K^+$ release) but also argues against the availability of low-affinity substrate sites except during sharply restricted segments of the reaction sequence, thereby favoring proposals that the low-affinity substrate sites are transformed into high-affinity substrate sites with the $E_2$ to $E_1$ conformational change.**

## Introduction

Essential to understanding the enzymatic and transport function of the $(Na^+ + K^+)$-dependent ATPase is the delineation of the reaction sequence and characterization of the ligand sites. Conflicting arguments persist, for example, about the number and nature of substrate sites and their response to monovalent cations [1–6]. An early study on $K^+$ and (millimolar) ATP kinetics suggested product release between addition of these ligands [7], and a later study measuring transport in erythrocytes indicated that $P_i$ was the product released [8]. Such experiments both (i) support a reaction sequence in which $K^+$ binds, $P_i$ is released, and ATP binds before $K^+$ is released, and (ii) make doubtful the simultaneous presence of $P_i$ and ATP on the enzyme [9].

Here we have reexamined the kinetics of cation and ATP interactions, in the absence and presence

* To whom correspondence should be addressed.

Abbreviations: Ado*PP*[NH]*P*, the $\beta$-$\gamma$ methylene analog of ATP; FITC, fluorescein isothiocyanate; $K^+$-phosphatase, $K^+$-dependent *p*-nitrophenylphosphatase; $(Na^+ + K^+)$-ATPase, $(Na^+ + K^+)$-dependent ATPase.

of spermine. Spermine has been reported to inhibit the $K^+$-dependent nitrophenylphosphatase reaction catalyzed by this enzyme, acting as a competitor toward $K^+$ [10–12], and, variously, either to stimulate the $(Na^+ + K^+)$-ATPase reaction, decreasing the $K_m$ for ATP [12], or to inhibit the $(Na^+ + K^+)$-ATPase reaction, acting as competitor to both $Na^+$ and $K^+$ [11]. In the current studies, effects of spermine on the $K^+$-phosphatase reaction are attributable to a parallel decrease in affinity for $Na^+$ and $K^+$, in terms of a previously proposed kinetic model [13]. Effects on the $(Na^+ + K^+)$-ATPase reaction are attributable to decreases in affinity for $Na^+$ as activator, for $K^+$ as product inhibitor, and, to a lesser extent, for $K^+$ as activator; using a steady-state model the effects of spermine on $K_m$ for ATP can be accounted for solely by these changes in affinity for cations. Spermine thus appears to be a specific and potentially revealing reagent, acting as a strong competitor at the cytoplasmically accessible $Na^+$ and $K^+$ sites and as a weaker competitor at the extracellularly accessible $K^+$ sites. Finally, consideration of this model also poses severe restrictions on the reaction sequence, particularly on the availability of the low-affinity substrate sites.

## Methods and Materials

The enzyme preparation was obtained from medullae of frozen canine kidneys, following the procedure of Jorgensen [14]. Specific activities in the standard medium (below) ranged from 15 to 22 $\mu$mol $P_i$ released/min per mg protein.

$(Na^+ + K^+)$-ATPase activity was measured at 37°C in terms of $P_i$ production, as described previously [7]. The standard incubation medium contained 30 mM histidine HCl-Tris (pH 7.8), 3 mM ATP, 3.5 mM $MgCl_2$, 90 mM NaCl and 10 mM KCl. $K^+$-phosphatase activity was measured at 37°C in terms of nitrophenol production, with nitrophenyl phosphate as substrate [15]. The standard incubation medium contained 30 mM histidine HCl-Tris (pH 7.8), 3 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$ and 10 mM KCl.

Data presented are averages of four or more experiments, each performed in duplicate or triplicate. Data were fitted by least-squares iterative methods to the Michaelis-Menten and Hill equations, using the KINFIT program [16], or empirically to specific formulations, including a ping-pong bi-bi scheme with product inhibition [17].

Frozen kidneys were obtained from Pel-Freeze, and ATP, *p*-nitrophenyl phosphate and spermine from Sigma.

## Results and Discussion

### *$K^+$-phosphatase reaction*

Spermine, at a concentration of 0.3 mM, inhibits $K^+$-phosphatase activity of the $(Na^+ + K^+)$-ATPase, increasing the $K_{0.5}$ for $K^+$ (Fig. 1), as reported earlier [10]. This effect of spermine can be accounted for by a previously proposed [13] model for the reaction (Fig. 2); assumptions implicit in this general scheme include competition between $Na^+$ and $K^+$ for binding to $E_1$ but with only $K^+$ favoring the conformational transition from $E_1$ to $E_2$, and significant binding of substrate only to $E_2$ conformations [13]. In this context, inhibition of phosphatase activity by spermine is attributable to a 2-fold decrease in affinity of $E_1$ for $K^+$ and a 4-fold decrease in affinity of $E_2 \cdot$ nitrophenyl phosphate for $K^+$ (Table I). The parameters fitted to the model were

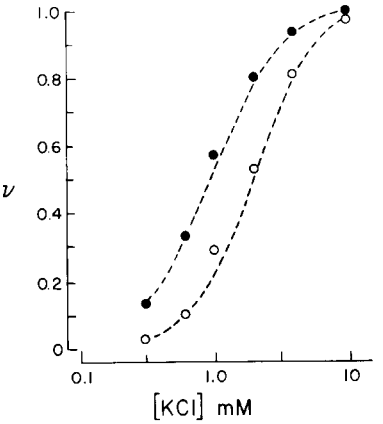


Fig. 1. Effects of spermine on $K^+$ activation of the $K^+$-phosphatase reaction. Enzymatic activity was measured in the standard incubation medium modified to contain the KCl concentrations shown, in the absence (●) or presence (○) of 0.3 mM spermine. Velocity is expressed relative to that in the unmodified medium in the absence of spermine, defined as 1.0. The dashed lines were calculated using the parameters of Table I in the equation of Fig. 2.

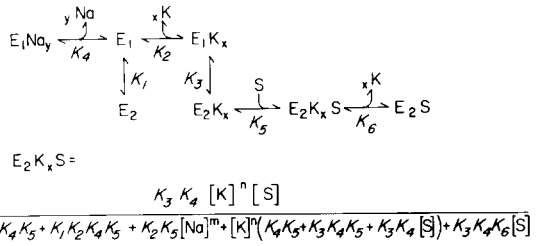

Fig. 2. Model for the $K^+$-phosphatase reaction. A rapid-equilibrium model for the pertinent enzyme conformations and ligand-bound states is shown, with velocity proportional to $E_2K_xS$ [13].

selected empirically, and optimal correspondence was not sought. Although the parameters chosen and presented are not unique [13], the relationship between them is highly constrained, as is the nature of the response to changes in these parameters (such as the proposed alterations attributed to spermine). The important features here are the particular parameters affected by spermine and the relative changes in their magnitude.

Raising the $MgCl_2$ concentration opposes inhibition by spermine (Table II), consistent with $Mg^{2+}$ competing with $K^+$ for the $K^+$ sites, as previously proposed [10] and suggested by other experimental approaches [18]. However, quantitative analyses of these interactions requires expansion of the model to include divalent cation binding, a major task that more than doubles the number of parameters, and is thus not attempted here.

TABLE I

VALUES OF PARAMETERS FOR MODEL OF $K^+$-PHOSPHATASE REACTION

These parameters were used in the equation of Fig. 2 to calculate the dashed lines of Figs. 1 and 3 and the $K_m$ values for substrate (in the text). Values were chosen empirically; units are scaled to millimolar concentrations.

| Parameter | Experimental conditions | |
|---|---|---|
| | control | with 0.3 mM spermine |
| $K_1$ | 0.7 | 0.7 |
| $K_2$ | 1.25 | 3.33 |
| $K_3$ | 5.0 | 5.0 |
| $K_4$ | 1.25 | 3.33 |
| $K_5$ | 2.22 | 2.22 |
| $K_6$ | 1.5 | 6 |
| $n$ | 1.55 | 1.8 |
| $m$ | 1.4 | 1.65 |

TABLE II

EFFECTS OF SPERMINE ON $K^+$-$Mg^{2+}$ INTERACTIONS

The enzyme preparation was incubated in the standard medium for assaying $K^+$-phosphatase activity, modified to contain the $MgCl_2$ and KCl concentrations shown, in the absence or presence of 0.3 mM spermine. Velocities are presented relative to that in the unmodified standard medium, defined as 1.0.

| $[MgCl_2]$ (mM) | [KCl] (mM) | Relative velocity | |
|---|---|---|---|
| | | control | with spermine |
| 1.0 | 10 | 0.71 | 0.72 |
| 1.0 | 1.0 | 0.70 | 0.24 |
| 3.0 | 10 | 1.00 | 0.99 |
| 3.0 | 1.0 | 0.57 | 0.48 |
| 15 | 10 | 0.98 | 0.97 |
| 15 | 1.0 | 0.19 | 0.23 |

$Na^+$ inhibits $K^+$-phosphatase activity (although stimulation can occur with very low $K^+$ concentrations [13,15]), and spermine affects this inhibition little (Fig. 3). Such a result would imply that changes in affinity for $Na^+$ be matched to the changes for $K^+$, and, indeed, the data are fitted by the model with a parallel decrease in affinity of $E_1$ for $Na^+$ (Table I). This similar decrease in affinity for both $K^+$ and $Na^+$ caused by spermine is thus sharply different from the changes in apparent affinity for cations caused by dimethyl sulfoxide and by oligomycin [13]: these reagents, respectively, increased and decreased the apparent affinity for $K^+$ by altering the equilibria between $E_1$ and $E_2$ conformations without affecting the actual binding to $E_1$, as interpreted by this model [13]. By contrast, effects of spermine are explainable without invoking alterations in the equilibrium constants for the conformational transition, and require a spermine-induced change in actual affinity.

It should be noted that the $K^+$ sites activating the phosphatase reaction are accessible from the cytoplasm [19], as are the $Na^+$ sites activating the ATPase reaction. An economical hypothesis identifies these sites with each other and with the sites at which $Na^+$ inhibits the phosphatase reaction. This identity is also supported by the parallel

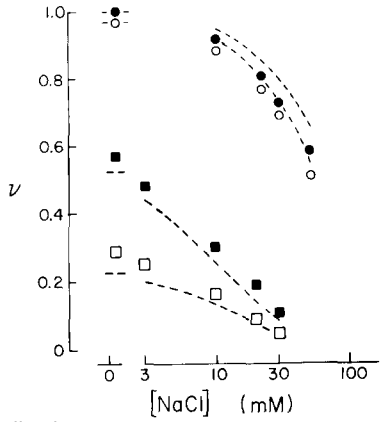


Fig. 3. Effects of spermine on inhibition by NaCl. $K^+$-phosphatase activity was measured in the standard incubation medium modified to contain the concentrations of NaCl shown and either 10 mM KCl (●, ○) or 1 mM KCl (■, □), in the absence (●, ■) or presence (○, □) of 0.3 mM spermine. Velocities are expressed as in Fig. 1, and the dashed lines were calculated using the parameters of Table I in the equation of Fig. 2.

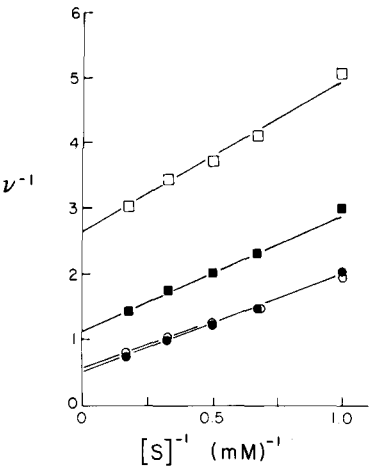


Fig. 4. Effects of spermine on substrate kinetics. $K^+$-phosphatase activity was measured in the standard incubation medium modified to contain the concentration of substrate, *p*-nitrophenyl phosphate, shown and either 10 mM KCl (●, ○) or 1 mM KCl (■, □), in the absence (●, ■) or presence ○, □) of 0.3 mM spermine. Velocities are expressed as in Fig. 1, and the data are presented in double-reciprocal form. The straight lines were fitted by the method of least squares, using the KINFIT program [16].

decrease in affinity for $Na^+$ and $K^+$ (Table I) caused by spermine, and suggests direct and specific interaction by this reagent.

The apparent $K_m$ for substrate, nitrophenyl phosphate, varies with KCl concentration [15], and the effects of spermine on the apparent $K_m$ at two KCl concentrations (Fig. 4) are also accommodated reasonably well by the model entirely in terms of changes in affinity for K: observed and calculated values, respectively, for incubations with 10 mM KCl alone are 2.9 and 2.6 mM; plus spermine, 2.5 and 2.5 mM; for incubations with 1 mM KCl alone, 1.6 and 1.4 mM; plus spermine, 0.9 and 0.7 mM. (With these parameters the fit of the maximal velocity, $V$ (with respect to substrate) in the presence of 1 mM KCl is, however, not quite as precise. In the absence of spermine, the observed $V$ with 1 mM KCl is 46% of that with 10 mM KCl, whereas the calculated $V$ is 40%; with 0.3 mM spermine the observed and calculated values are, respectively, 21% and 16%.) Again, effects of spermine only on cation sites need be invoked.

### *$(Na^+ + K^+)$-ATPase reaction: studies with $Na^+$ and $K^+$*

Experiments described in the previous section suggests that for the $(Na^+ + K^+)$-ATPase reaction

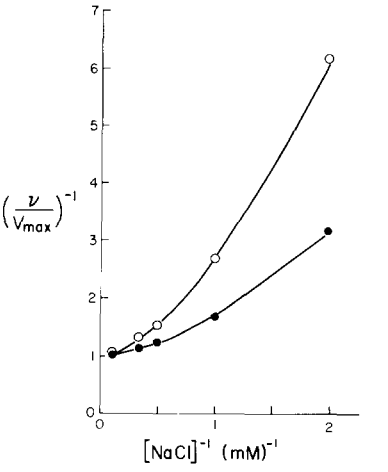


Fig. 5. Effects of spermine on $Na^+$ activation of the $(Na^+ + K^+)$-ATPase reaction. Enzymatic activity was measured in the standard incubation medium modified to contain the concentrations of NaCl shown, in the absence (●) or presence (○) of 0.3 mM spermine. Data are presented in double-reciprocal form. The curved lines were fitted by the method of least squares to the Hill equation, using the KINFIT program [16]. Calculated $K_{0.5}$ values are 8 mM (control) and 14 mM (spermine); the calculated Hill coefficients are 1.63 (control) and 1.59 (spermine).

spermine should decrease the affinity for $Na^+$ at its activating sites (identified with the sites at which $Na^+$ inhibits the $K^+$-phosphatase reaction) and should decrease the affinity for $K^+$ at its cytoplasmically accessible discharge sites (identified with the sites at which $K^+$ activates the $K^+$-phosphatase reaction). In accord with the first tenet of this formulation, spermine increase the $K_{0.5}$ for $Na^+$ nearly 2-fold, from 8 mM to 14 mM (Fig. 5). This result is in good agreement with the effects of spermine on the $K^+$-phosphatase reaction, in light of competitions between $K^+$ and $Na^+$ for their respective sites [20] and the difference between steady-state determinations of $K_{0.5}$ and actual dissociation constants.

With regard to the second tenet of the formulation, a decrease in affinity for $K^+$ at its discharge sites, this proposal is not directly measurable in such enzymatic studies, but it can be approached in terms of the steady-state kinetic model (see below).

Effects of spermine on $K^+$-activation can, however, be directly assessed (although the results are inevitably complicated by such factors as competitions between monovalent cations for their activating sites and by $K^+$ also acting as a product inhibitor). Spermine increased the $K_{0.5}$ for $K^+$ from 0.8 mM to 1 mM (fig. 6), a much smaller change than for $Na^+$.

*($Na^+ + K^+$)-ATPase reaction: studies with ATP*

In the absence of spermine, double-reciprocal plots of velocity vs. ATP concentration are linear over the range 0.3–3 mM (Fig. 7), corresponding to occupancy of the low-affinity substrate sites [21,22]. When such experiments are repeated with different fixed levels of KCl, in the range 1–10 mM, a family of parallel lines results (Fig. 7), as originally noted [7] and subsequently confirmed [23].

Such a kinetic pattern is consistent with a ping-pong reaction sequence of first substrate addition, product relase (which is an essentially irreversible step under initial velocity conditions, since no appreciable amount of product is then available to recombine with the enzyme), and second substrate addition. Here the two substrates are $K^+$ and ATP, and the possible products are $P_i$ and ADP (release of $Na^+$ and $K^+$, also products, cannot so serve since they are present in the medium and thus product release of these ligands is not irreversible). More recently, Eisner and

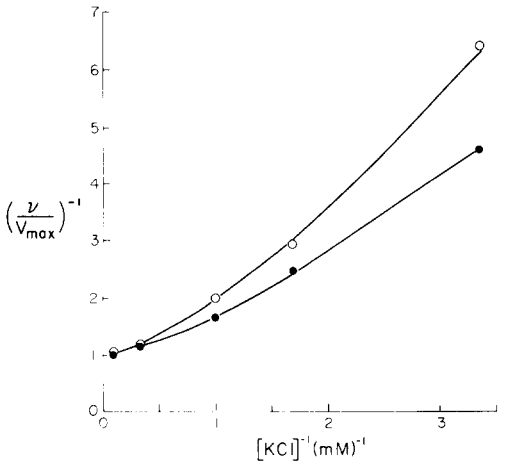


Fig. 6. Effects of spermine on $K^+$ activation. Experiments were performed and the data are presented as in Fig. 5 except that the KCl concentrations were modified as shown. Calculated $K_{0.5}$ values are 0.8 mM (control) and 1.0 mM (spermine); the calculated Hill coefficients are 1.37 (control) and 1.41 (spermine).

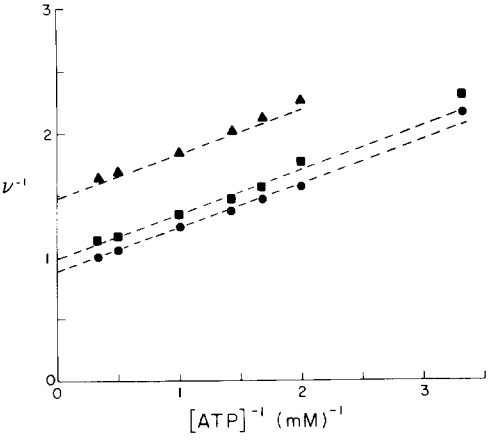


Fig. 7. $K^+$-ATP interactions in the ($Na^+ + K^+$)-ATPase reaction. Enzymatic activity was measured in the standard medium modified to contain the concentrations of ATP shown, with the $MgCl_2$ concentration kept 0.5 mM greater than the ATP concentration, in the presence of 10 (●), 3 (■), or 1 mM (▲) KCl. Velocities are expressed relative to that in the standard incubation medium defined as 1.0, and data are presented in double-reciprocal form. The dashed lines were calculated using the parameters of Table III in the equation of Fig. 8.

Richards [8] showed that, in the complementary experiment varying the $K^+$ analog, $Rb^+$, at fixed levels of ATP, addition of $P_i$ converted parallel plots to intersecting plots. This finding confirms the likelihood that $P_i$ release is the step between substrate additions. Since early studies on the enzyme demonstrated that the major release of $P_i$ follows $K^+$ addition [24], the reaction sequence must be: $K^+$ binds to its activating sites, $P_i$ is released from the enzyme, and then ATP binds to the low-affinity substrate sites. It should be emphasized that under these experimental conditions the extent of ATP-binding to the phosphorylated enzyme must be quite low, or else the parallel plots would be transformed into intersecting plots. Consequently, even if the low-affinity sites are modulating sites distinct from the high-affinity substrate sites through which enzyme phosphorylation occurs, their occupancy is limited to only brief segments of the reaction cycle.

For current purposes the reaction sequence can be compressed into four steps of a ping-pong bi-bi steady-state kinetic system [17], representing $K^+$ addition, $P_i$ release, ATP addition, and $K^+$ release (Fig. 8). Such compression is justifiable under these experimental conditions either because an interaction does not occur (e.g., product inhibition by ADP, since ADP levels are negligible), or because a ligand is held constant and its contribution to the rate constants not altered (e.g., the $Na^+$ concentration in these experiments is not varied and is nearly saturating at its intracellularly accessible activating sites, and thus the consequences of its acting both as activator and product inhibitor are essentially unchanged in this set of experiments; however, effects of spermine on the Na sites will be invoked, below, although this level of $Na^+$ interactions is maintained).

This model ignores $Na^+$-ATPase activity of the enzyme, which is considered in conventional Albers-Post formulations to reflect an alternative reaction pathway in the absence of $K^+$, one with a $K_m$ for ATP comparable to that of the high-affinity substrate sites of the $(Na^+ + K^+)$-ATPase pathway and three orders of magnitude lower than the $K_m$ being studied here [1–3]. The contribution of this pathway to the current experiments, and its influence on their analysis, is considered minor since (a) the ATP concentration is maintained in

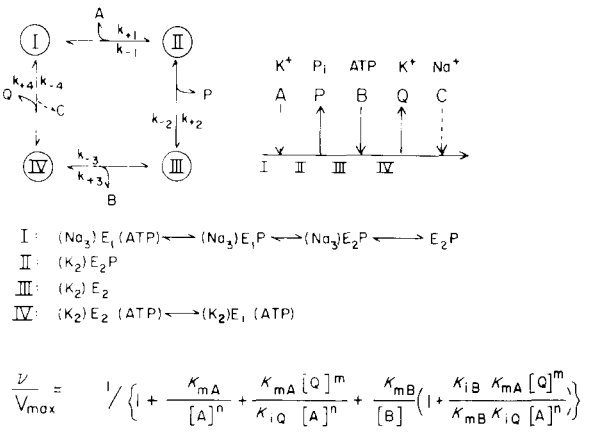


Fig. 8. Steady-state kinetic model for the $K^+$-ATP interactions of the $(Na^+ + K^+)$-ATPase reaction. $K^+$ and ATP are depicted as first (A) and second (B) substrate of a ping-pong bi-bi reaction scheme, with $P_i$ the first (P) and $K^+$ the second (Q) product. Cooperative interactions at the two classes of $K^+$ sites are indicated with Hill constants $n$ and $m$. The reaction sequence is collapsed into four steps, I, II, III, IV, related by the standard parameters [17]; ($Na^+$ addition is shown as a third substrate, but ADP release and $Na^+$ release are not indicated.)

all experiments far above the $K_m$ for the $Na^+$-ATPase pathway, (b) the $Na^+$ concentration is held constant at a high level, and (c) with this range of KCl concentrations the $Na^+$-ATPase activity is only several percent of the total activity with $Na^+$ plus $K^+$.

With this scheme the experimental data can be fitted (Fig. 7) with reasonable values for the parameters (Table III). Again, the parameters were

TABLE III

VALUES OF PARAMETERS FOR MODEL OF $(Na^+ + K^+)$-ATPase REACTION

These parameters were used in the equation of Fig. 8 to calculate the dashed lines of Figs. 7 and 9. Values were chosen empirically.

| Parameter | Experimental conditions | |
|---|---|---|
| | Control | with spermine 0.3 mM |
| $K_{mA}$ | 0.9 | 1.1 |
| $K_{mB}$ | 0.5 | 0.25 |
| $K_{iB}$ | 0.1 | 0.1 |
| $K_{iQ}$ | 4.0 | 16.0 |
| $n$ | 1.4 | 1.4 |
| $m$ | 1.4 | 1.4 |

evaluated empirically, and although not unique are highly constrained by the data and by the equation in their relationship to each other. It should also be noted that the parallel form of response (Fig. 7) prohibits significant $K^+$ release before ATP binding, as well as direct competition between $K^+$ and ATP binding to the low-affinity substrate sites, previously proposed [25].

The kinetic data incorporated in this scheme are thus consistent with a role for ATP in promoting $K^+$-release [27,28], at least under conditions within this range of ligand concentrations. The most economical hypothesis is that the low-affinity substrate sites are transformed into high-affinity substrate sites with the $E_2$ to $E_1$ conformational change [4,29]. The opposite transformation, $E_1$ to $E_2$, would thus include a distortion of the substrate site, involving misalignment of the FITC-binding site (reflecting the region where adenine binds [30]), and the phosphate acceptance site (adjacent to the gamma-phosphate of ATP at the high-affinity site); in the primary sequence these two regions are quite far apart [31,32]. Such distortion thus could allow ATP binding at the low-affinity site in the presence of phosphate or vanadate bound to the enzyme [27,28], even if such occupancy does not occur to an appreciable extent under usual conditions of $(Na^+ + K^+)$-ATPase assays. One indicator of the extent of distortion in the low-affinity substrate site is the loss of ATP function through that site after treatment with FITC [33], attributable to loss of the adenine binding site, vs. the retention of phosphatase activity not only with the relatively small substrate nitrophenyl phosphate [33] but also with the far bulkier substrates umbelliferone phosphate and 3-*O*-methylfluorescein phosphate (Robinson, J.D., unpublished observations).

These considerations, however, leave uncertain the functional significance of the low-affinity binding sites for Ado*PP*[NH]*P* [5]. If these represent the low-affinity substrate sites, then the kinetic argument above requires that their occupancy by ATP is restricted to those steps in the reaction sequence after $P_i$ release and before $K^+$ addition.

*$(Na^+ + K^+)$-ATPase: studies with ATP and spermine*

In the presence of spermine, double-reciprocal plots of velocity vs. ATP concentration at several fixed $K^+$ concentrations remain a family of parallel lines (Fig. 9), although they are rotated clockwise and spread somewhat further apart compared to the control plots (Fig. 7). Spermine thus can stimulate $(Na^+ + K^+)$-ATPase activity at low ATP concentrations and high KCl (and NaCl) concentrations, as previously reported [10,12], or inhibit at low KCl (and NaCl) concentrations, as also previously reported [11]: these observations therefore reconcile those disparate findings.

More important is interpreting the effects of spermine within the ping-pong kinetic model (Fig. 8), for this bears on the general validity of that model as well as on the specificity of the spermine interactions, which with the $K^+$-phosphatase reaction could be limited to changes in affinity at cytoplasmic monovalent cation sites (above). To fit the ATPase data in the presence of spermine to the model requires changing a minimum of three parameters (Table III), $K_{iQ}$, $K_{mA}$, and $K_{mB}$.

Of these, the change in $K_{iQ}$ is most obvious in light of the experiments on the phosphatase reaction. $K_{iQ}$ represents in this context release of $K^+$ from the sites identified above with those activating the $K^+$-phosphatase reaction. Consequently, a spermine-induced decrease in affinity for $K^+$ at the activating sites for the phosphatase reaction

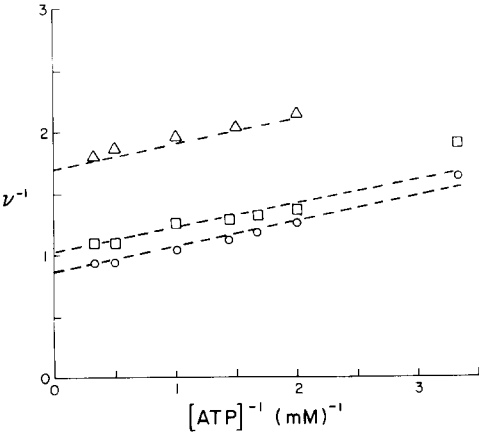


Fig. 9. $K^+$-ATP interactions in the presence of spermine. Experiments were performed and data are presented as in Fig. 7, except that 0.3 mM spermine was present in the incubation media. Again, the dashed lines were calculated using the parameters of Table III in the equation of Fig. 8.

would correlate with a spermine-induced decrease in affinity for $K^+$ at the discharge sites in the ATPase reaction, i.e., with an increase in $K_{iQ}$. Since $K_{iQ} = k_4/k_{-4}$ [17], an increase in $K_{iQ}$ is consistent with a decrease in $k_4$, the rate at which $K^+$ binds to the discharge site. However, in compressing the reaction sequence to the four steps of Fig. 8, the rate constant for $K^+$ release, $k_4$, is conflated with those for other steps, including the rate constants for $Na^+$ binding and $Na^+$ release. Spermine would thus be expected, from the effects on $Na^+$ affinity noted in the model for the phosphatase reaction (Fig. 2; Table I) and on the $K_{0.5}$ for activation of the ATPase reaction (Fig. 5), also to decrease $k_4$ somewhat. The requirement that $k_{-4}$ be decreased by spermine more than $k_4$ to generate the 4-fold increase in $K_{iQ}$ is not inconsistent with these aspects of the reaction sequence, since a lesser inhibition of $Na^+$ discharge from extracellular sites (see below), where $Na^+$ is released, would tend to offset within $k_4$ the greater inhibition of binding of $Na^+$ to cytoplasmic sites. Plausible effects of spermine, then, would be to decrease $k_4$ 2-fold and $k_{-4}$ 8-fold.

$K_{mA}$, representing binding of $K^+$ to its extracellularly oriented acceptance sites, is changed far less. $K_{mA}$ is equal to $k_3k_4k_p$ divided by $k_1$ times a collection of kinetic terms [17]. If spermine is to affect cation binding solely, then changes in $k_3$ and $k_p$ are ruled out by hypothesis. An increase in $K_{mA}$ thus requires that $k_4$ increase and/or $k_1$ decrease. The likelihood that spermine decreases $k_4$ was raised in the preceding paragraph, so $k_1$ must be decreased even more if $K_{mA}$ is to increase. Nevertheless, assuming a 2-fold decrease in $k_4$ (which includes, among other steps, $Na^+$ binding and $Na^+$ release), then to increase $K_{mA}$ from 0.9 to 1.1 would require roughly a 2.5-fold decrease in $k_1$, the rate constant for binding of $K^+$ to its acceptance sites. This is clearly far smaller than the decrease in $k_{-4}$, the rate constant for binding of $K^+$ to its discharge sites, needed to increase $K_{iQ}$ 4-fold (i.e., if $k_4$ is decreased 2-fold, then $k_{-4}$ must be decreased 8-fold). Spermine would thus appear to affect the extracellularly oriented $K^+$ sites far less than the cytoplasmically oriented $K^+$ sites (e.g., 2.5-fold vs. 8-fold). Spermine binding at or near the cytoplasmic sites would be expected to influence them far more strongly than the extracellular aspects of the transport mechanism.

$K_{mB}$ relates to ATP binding, but spermine-induced changes need not represent changes in actual affinity for ATP (represented by $K_{iB}$), since $K_{mB}$ is equal to $k_4$ in the numerator (times a series of kinetic terms) divided by $k_2$ in the denominator (times a series of kinetic terms). The 2-fold decrease in $k_4$ suggested above would thus alone account for the 2-fold decrease in $K_{mB}$ (Table III), without requiring any change in affinity for ATP.

These considerations of the kinetic model illustrate that spermine-induced changes in the $(Na^+ + K^+)$-ATPase reaction, manifested by alterations in $K_{0.5}$ for $Na^+$ and $K^+$ and in $K_m$ for ATP, and either stimulation or inhibition of the ATPase reaction, can be reconciled with the data on spermine inhibition of the $K^+$-phosphatase reaction. The only action required beyond inhibition of $Na^+$ and $K^+$ binding to their cytoplasmic sites is a (lesser) inhibition of $K^+$ binding to its extracellular sites.

## Conclusions

Experiments with spermine on the $K^+$-phosphatase reaction support an earlier model [13] for that reaction process, and, when interpreted in terms of that model, suggest that spermine affects cation and substrate interactions by decreasing affinity for $Na^+$ and $K^+$, in parallel, at their cytoplasmically-accessible sites.

Experiments with spermine on the $(Na^+ + K^+)$-ATPase reaction are consistent with decreased affinity for $Na^+$ and $K^+$ at their cytoplasmically accessible sites, but, when interpreted in terms of a ping-pong steady-state model, require in addition a lesser decrease in affinity for $K^+$ at its extracellularly accessible sites. These changes in affinity account for spermine-induced alterations in $K_{0.5}$ for activating $Na^+$ and $K^+$ and in $K_m$ for ATP, and resolve the earlier discrepancy between spermine-induced inhibition vs. stimulation. Moreover, spermine appears to be a potentially useful tool for exploring further the monovalent cation sites and their effects.

Considerations of $K^+$-ATP interactions not only support a specific reaction sequence ($K^+$ addition,

$P_i$ release, ATP addition, $K^+$ release), but argue against low-affinity substrate site availability except during restricted segments of the reaction sequence. This argument strongly supports interconversion of low- and high-affinity substrate sites, and severely limits the possibilities for functional interaction between catalytic subunits in oligomeric enzyme complexes.

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