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CATION SITES OF THE ($Na^+ + K^+$)-DEPENDENT ATPase:

MECHANISMS FOR Na^+-INDUCED CHANGES IN K^+ AFFINITY OF THE PHOSPHATASE ACTIVITY

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SUMMARY

Monovalent cation interactions with the K^+-dependent phosphatase activity of a rat brain ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparation were reexamined in attempts to distinguish between two models designed to account for stimulation of phosphatase activity by Na^+ when K^+ concentrations are low, but inhibition when K^+ concentrations are high. One model (Robinson, J. D. (1970) *Arch. Biochem. Biophys.* 139, 17–27) proposes that in the presence of Na^+ an alternative enzyme pathway, characterized by a higher affinity for K^+, becomes available through the phosphorylation of the enzyme by substrate. The second model (Albers, R. W. and Koval, G. J. (1973) *J. Biol. Chem.* 248, 777–784) attributes the response to distinct classes of sites for K^+, without involving the participation of substrate. Data obtained using umbelliferone phosphate can be fitted quantitatively to the original model. In addition, experiments evaluating K^+ affinity through a Be^{2+} inactivation technique demonstrated that both Na^+ and substrate were necessary for the increase in K^+ affinity. Similar experiments with nitrophenyl phosphate and a non-phosphorylating analog that can attain the active site, nitrobenzyl phosphonate, imply that phosphorylation was the necessary factor. These data support the original proposal, but do not rule out the possibility that, in addition, there may be multiple classes of sites for K^+.

INTRODUCTION

Because the ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) is intimately associated with the cellular mechanism for transporting Na^+ and K^+ (refs 1–3), the activation of the enzyme by these cations has attracted particular interest. Among various approaches to this problem considerable attention has focussed on cation activation of a K^+-dependent phosphatase activity present in ATPase preparations, for this activity apparently reflects the terminal hydrolytic

steps of the overall ATPase reaction[1-3]. Examination of this phosphatase activity thus permits access to a seemingly less complex reaction process, and also affords opportunities for studying K^+ interactions in the absence, as well as presence, of Na^+. From such studies have come data on K^+ activation consistent with multiple (but equivalent) K^+ sites exhibiting cooperative homotropic interactions[4-7].

In the presence of Na^+, however, activation of the phosphatase by K^+ appears less straightforward: although Na^+ inhibits at higher K^+ concentrations, at low concentrations of K^+ it stimulates phosphatase activity[4,8]. The realization that this anomalous stimulation by Na^+ could not be accomodated within previous schemes prompted the proposal[5,6] that, in the presence of Na^+, an alternative enzymatic pathway for phosphatase activity becomes available: a portion of the enzyme is transformed from the form that hydrolyzes the substrate over the conventional phosphatase pathway, reflecting the terminal hydrolytic stages of the ATPase, to an alternative enzyme form capable of hydrolyzing the phosphatase substrate over the total ATPase pathway. This second pathway would then reflect steps of the reaction sequence involving Na^+-dependent phosphorylation of the enzyme, followed by K^+-dependent hydrolysis of this acyl phosphate. Consequently, in the presence of Na^+ two pathways would be available, with enzymatic traffic divided according to the concentrations of Na^+ and K^+. This proposal was supported by showing that in such a system qualitatively similar velocity–activator curves could be obtained[5]. Subsequently, preliminary data were presented demonstrating Na^+-dependent phosphorylation of the enzyme by a phosphatase substrate, nitrophenyl phosphate, which was sensitive to hydroxylamine[9].

Recently, Albers and Koval[10] re-examined the kinetics of the K^+-dependent phosphatase in the presence of Na^+, and, without considering the above model, proposed an alternative scheme requiring that there be at least two distinct classes of K^+ sites. A computer-generated curve-fitting program demonstrated a reasonably good quantitative agreement between data and model.

To reassess the validity of the original alternative-pathway model it thus seemed necessary to determine if a quantitative fit of the data could be made to this model as well. But obviously mere agreement between data and model cannot establish the validity of any proposal. Therefore a second approach to resolve this issue was undertaken in which the affinity of the enzyme for K^+ can be measured in various ligand states. The original model requires that substrate be present to effect the change in K^+ affinity, through creation of the alternative enzymatic pathway (requiring Na^+-dependent phosphorylation of enzyme by substrate); on the other hand, the Albers–Koval model does not, in the form proposed, involve participation of substrate.

METHODS AND MATERIALS

The $(Na^+ + K^+)$-dependent ATPase was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described[11].

K^+-dependent umbelliferone phosphatase activity was measured fluorimetrically[12] in terms of umbelliferone production during incubation of the enzyme at 37 °C in a standard control medium containing 30 mM histidine–Tris (pH 7.8), 3 mM $MgCl_2$, 1 mM umbelliferone phosphate (as the Tris salt), 20 mM KCl, and 10% di-

methylsulfoxide (v/v). K^+-independent activity, which averaged a few percent of the total activity measured in the standard medium, was measured concurrently in parallel incubations omitting KCl; this activity was subtracted from the total to give K^+-dependent activity.

Enzymatic activity was either monitored continually during incubation in the fluorimeter cuvette[12] in experiments to determine the time course, or, routinely, after brief incubations (10–15 min) during which activity had previously been found to be linear with time. In this latter case activity was halted by adding trichloroacetic acid to a final concentration of 5% (w/v), the mixture centrifuged, and an aliquot of the supernatant material added to 5 vol. of 1.0 M Tris. Fluorscence was measured with an Aminco–Bowman spectrophotofluorimeter equipped with a ratio-recording accessory; excitation was at 370 nm and emission measured at 460 nm.

K^+-dependent nitrophenyl phosphatase activity was measured analogously, in terms of nitrophenol production, as described previously[4]. The standard control medium was identical to that described above, except that 3 mM *p*-nitrophenyl phosphate (as the Tris salt) was substituted for umbelliferone phosphate.

The affinity for K^+ was approached in terms of K^+-dependent inactivation of the (Na^+ + K^+)-dependent ATPase activity by $BeCl_2$, as previously described in detail[13]. In brief, these experiments involved determining the pseudo first-order rate constants for enzyme inactivation as a function of K^+ concentration, by means of initial incubations at 37 °C of enzyme (0.5 mg protein/ml), 10% dimethylsulfoxide (v/v), 250 μM $BeCl_2$, 3 mM $MgCl_2$, and a range of KCl concentrations (*plus* other additions as specified). These inactivating incubations were terminated by adding 4 vol. of a standard ATPase incubation medium and the residual activity then measured during brief incubations (changes in activity during these latter incubations were negligible since the inactivation is essentially irreversible and the added NaCl blocks further inactivation). Kinetically, the resultant pseudo first-order rate constants, k_{in}, can be treated analogously to initial velocities in enzyme kinetics. Consequently, $1/k_{in}$ plotted against $1/[K^+]$ will provide an index of K^+ affinity to the sites controlling inactivation by Be^{2+}; previous arguments identify these with the K^+-activating sites of the ATPase and phosphatase[13].

p-Nitrophenyl phosphate and ATP were purchased from Sigma Chemical Co., and umbelliferone phosphate from Isolabs, Inc. *p*-Nitrobenzyl phosphonate was synthesized according to Kosolapoff[14].

The data presented are averages of five or more experiments performed in duplicate; enzymatic activity is expressed relative to that of concurrent controls incubated in the standard medium. Curve fitting was performed by an empirical approach using an SEL-810A computer, and the lines in Figs 1 and 2 were obtained from Eqn 4 by a Calcomp plotter provided with the parameters of Table I and the appropriate ranges for Na^+ and K^+.

RESULTS AND DISCUSSION

K^+-dependent phosphatase activity was measured using umbelliferone phosphate as substrate: to extend the previous studies with nitrophenyl phosphate[4–8,10], and to permit continuous monitoring of the reaction time course (see Methods and Materials). Dimethylsulfoxide, 10% (v/v), was included in all incubations since, in

accord with the report of Albers and Koval[10], the stimulation by Na^+ at low K^+ concentrations was thereby accentuated.

Under these conditions activation of the K^+-dependent umbelliferone phosphatase followed the kinetic patterns previously described with nitrophenyl phosphate[4–8,10]. In the absence of Na^+ the activator–velocity plot was sigmoidal, the double-reciprocal plot was concave upward (Fig. 1), and the Hill plot had a slope, n,

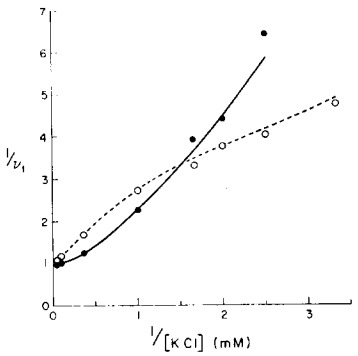

Fig. 1. K^+ activation of the umbelliferone phosphatase activity. The enzyme preparation was incubated in the standard medium (see Methods and Materials), but with the concentrations of KCl indicated, in the absence (●) or presence (○) of 10 mM NaCl. Enzyme velocity (v_t) is expressed relative to that in the standard medium without modification, defined as 1.0, and the data are presented in double-reciprocal form. Lines were drawn by a Calcomp plotter from Eqn 4, using the constants from Table I and the appropriate values of Na^+ and K^+.

greater than 1.0. Such a kinetic pattern is consistent with cooperative interactions within a single class of K^+ sites[4–7]. By contrast, in the presence of Na^+ the kinetic response was complex (Fig. 1), with this response to Na^+ being dependent on K^+ concentration (Fig. 2).

Such responses represent less straightforward kinetic processes, and the quantitative fit of these data may be considered in terms of the alternative-pathway model originally proposed[5,6]. Assume that, in the presence of Na^+, a fraction of the enzyme is converted to a new form so that now two parallel pathways are available, the first being the conventional phosphatase pathway as routinely measured in the

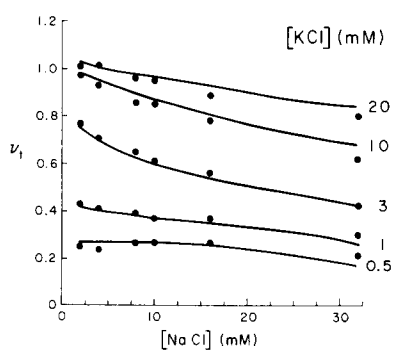

Fig. 2. Effects of NaCl on umbelliferone phosphatase activity at various KCl concentrations. Experiments were performed as in Fig. 1, with the concentrations of NaCl and KCl indicated. Lines were drawn from Eqn 4, as in Fig. 1.

absence of Na^+, and the second being a modified pathway occurring in the presence of Na^+ and a substrate able to form an acyl phosphate intermediate (such as certain nucleotides[4,15] and to a lesser extent phosphatase substrates[9,16]). The latter enzyme form and pathway is characterized by having a marked increase in apparent affinity for K^+ (refs 4, 13, 17, 18).

Under these circumstances the observed total velocity, v_t, the sum over both pathways, is

$$v_t = v_a + v_b \tag{1}$$

where v_a and v_b are velocities over each pathway. More specifically,

$$v_a = \frac{(1-f)V_1}{1+\left[\frac{K_1}{[K^+]}\left(1+\frac{[Na^+]}{K_2}\right)\right]^{n_1}} \quad ; \quad v_b = \frac{fV_2}{1+\left[\frac{K_3}{[K^+]}\left(1+\frac{[Na^+]}{K_4}\right)\right]^{n_2}} \tag{2}$$

where V_1 and V_2 are the maximal velocities for each enzyme pathway (representing the two enzyme forms); K_1 and K_3 are the affinities for K^+ for each form; and K_2 and K_4 are the inhibitory constants for Na^+ competition with K^+ for the K^+ sites. These are in the form of Hill equations for enzyme activity, incorporating competitive inhibition as formalized by Albers and Koval[10]; n_1 and n_2 are the Hill coefficients representing the extent of cooperative interaction between K^+ sites. The fractional portion of the total enzyme in form b, the phosphorylated enzyme, is designated f; consequently that fractional portion in the conventional form, a, is $(1-f)$.

The fraction, f, of the enzyme in the phosphorylated form will be a function of the Na^+ concentration (necessary to permit phosphorylation), the K^+ concentration (as a competitor toward Na^+ for the Na^+ sites), and the concentration of phosphorylating substrate. Let P equal the maximal fraction of enzyme in form b for a given level of substrate. Then the actual extent of phosphorylation, the extent of conversion to form b, will be given by:

$$f = \frac{P}{1+\left[\frac{K_5}{[Na^+]}\left(1+\frac{[K^+]}{K_6}\right)\right]^{n_3}} \tag{3}$$

where K_5 is the affinity for Na^+ at the sites controlling phosphorylation, K_6 is the inhibitory constant for K^+ at these sites, and n_3 is the index of cooperative interactions between these sites.

Combining equations 1–3 the observed velocity would be:

$$v_t = \frac{V_1\left\{1-\frac{P}{1+\left[\frac{K_5}{[Na^+]}\left(1+\frac{[K^+]}{K_6}\right)\right]^{n_3}}\right\}}{1+\left[\frac{K_1}{[K^+]}\left(1+\frac{[Na^+]}{K_2}\right)\right]^{n_2}} + \frac{\frac{PV_2}{1+\left[\frac{K_5}{[Na^+]}\left(1+\frac{[K^+]}{K_6}\right)\right]^{n_3}}}{1+\left[\frac{K_3}{[K^+]}\left(1+\frac{[Na^+]}{K_4}\right)\right]^{n_4}} \tag{4}$$

where the denominator of each term represents the fraction of maximal velocity due to K^+ activation, as reduced by Na^+ competition, and the numerator of each term reflects the division of total enzyme between these two forms.

Although the final expression is moderately involved, the physical model is straightforward. The substrate can be hydrolyzed by two routes with the division

of traffic a function of certain enzyme ligands. In the simplest case, the absence of Na^+, all activity proceeds over pathway a (Fig. 1). With increasing concentrations of Na^+ more enzyme is in form b and more substrate is transformed over this pathway, which is characterized by a markedly greater affinity for K^+.

Given an equation with 12 constants it is not surprising that it can be fitted to a set of data. However, to achieve a fit with reasonable values for these constants constrains the choices. With an empirical approach to curve-fitting it was not realistic to attempt an optimal congruence; instead, an effort was made to achieve a good approximation using values for these constants close to those previously reported. Values for V_1, K_1, and n_1 were estimated from the special case of the conventional pathway in the absence of Na^+ (Fig. 1), and K_2 from inhibitory constants[4,13]. Previous experiments indicated that the affinity for K^+ of the phosphorylated enzyme, form b, is roughly 20-fold greater than that of the conventional pathway[13]; no great change in Na^+ affinity seems apparent, and the index of cooperativity is perhaps less[4]. Maximal velocity over the alternative pathway, V_2, is near that of V_1 (ref. 4). For phosphorylating the enzyme in the absence of K^+ a $K_{0.5}$ for Na^+ around 2 mM has been observed[19], and it is stated that the affinity of these sites for K^+ is one-fourth to one-fifth as great as that for Na^+ (ref. 3). Evaluation of P is by guess, although previous studies with differing concentrations of phosphorylating substrate[4] indicate that it can be far less than 1.0 when marked effects on K^+ affinity are noted. Thus values were selected within these limits (Table I) that provide a remarkably good fit to the observed data: the lines of Figs 1 and 2. Obviously this model will accomodate the data as well as the alternative recently proposed[10].

TABLE I

KINETIC CONSTANTS FOR Eqn 4

Constants were selected from ranges of plausible values suggested in the literature (see text) and from an empirical curve-fitting approach to the data of Figs 1 and 2. Units are mM for activating and inhibitory constants (K values) and in arbitrary units for maximal velocities; n and P are dimensionless.

Constant	*Value*	*Constant*	*Value*
K_1	1.3	V_1	1.07
K_2	5.0	V_2	0.9
K_3	0.07	n_1	1.4
K_4	4.0	n_2	1.1
K_5	2.0	n_3	1.2
K_6	10	P	0.45

But to distinguish between these proposals, the alternative-pathway model[5,6] *vs* the multiple-classes-of-sites model[10], fitting data to kinetic equations is clearly inadequate, and other approaches are required. Recently an approach to measuring affinity constants for K^+ has been described[13] in terms of K^+ modification of ATPase inactivation by Be^{2+}. This kinetic treatment deals with the pseudo first-order rate constants for inactivation, k_{in}, as a function of K^+ concentration, presented in double-reciprocal form, since the kinetic analysis is formally equivalent to enzyme kinetics. Consequently K_{in}, the pseudo first-order rate constant at infinite K^+ concentration, is obtained from the reciprocal of the intercept on the ordinate, and $K_{0.5}$, the concentration to achieve half K_{in}, from the reciprocal of the projection on the

abscissa at half K_{in}. The latter is a measure of the affinity for K^+ at the sites modifying Be^{2+} inactivation and enzyme activity[13]. This method, therefore, permits estimation of the affinity for K^+ not only in the presence of substrate (as is required in routine enzymatic approaches) but also in its absence.

With this technique it is apparent that in the presence of enzyme, Mg^{2+}, and K^+ (*plus* Be^{2+} for inactivation) Na^+ acted merely as a competitor (Fig. 3): apparent affinity for K^+, as reflected in the value of $K_{0.5}$, was decreased whereas K_{in} was unchanged. In the presence of the substrate, umbelliferone phosphate, the kinetic

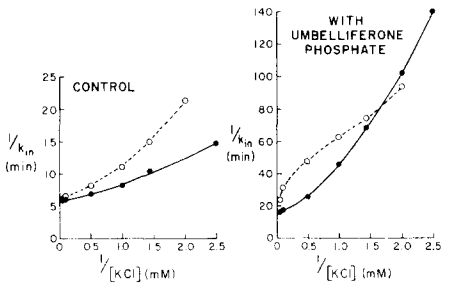

Fig. 3. Effects of K^+, Na^+ and umbelliferone phosphate on inactivation by Be^{2+}. Pseudo first-order rate constants were obtained for inactivation of the ($Na^+ + K^+$)-dependent ATPase activity, as described under Methods and Materials. The inactivating incubations were in media containing 30 mM histidine–Tris (pH 7.8), 3 mM $MgCl_2$, 250 μM $BeCl_2$, 10% (v/v) dimethylsulfoxide, and the concentrations of KCl indicated, in the absence (●) and presence (○) of 10 mM NaCl. In the right-hand panel are shown results of similar experiments, in which the media contained in addition 1.0 mM umbelliferone phosphate. Pseudo first-order rate constants for inactivation, k_{in}, are plotted against the KCl concentration in double-reciprocal form. Lines were fitted to the experimental points by eye.

response remained uncomplicated when Na^+ was absent: K_{in} decreased (as also noted in the presence of nucleotides[13]), possibly through chelation of Be^{2+}, and $K_{0.5}$ for K^+ is perhaps increased slightly. But now the addition of Na^+ strikingly transformed the kinetic response (Fig. 3) to the pattern seen with the phosphatase activity in the presence of Na^+ and K^+ (Fig. 1). Clearly the substrate is required to effect the transformation in the presence of Na^+. From crude extrapolation it is apparent that both portions of the kinetic plot, at high and at low K^+ concentrations, would give apparent affinities reasonably near those for the phosphatase activity (Fig. 1 and Table I), but a quantitative curve-fitting approach using Eqn 4 did not seem warranted; among other considerations the value of P is doubtlessly altered in the presence of Be^{2+}. Nevertheless, these data demonstrate that the Na^+-induced increase in affinity for K^+ (seen as stimulation of the phosphatase activity at low K^+ concentrations), is dependent on the presence of substrate, as required by the alternative-pathway model.

To determine if phosphorylation of the enzyme by substrate is necessary, as is also required by the model, similar experiments were performed with both *p*-nitrophenyl phosphate, the phosphatase substrate that is also reported to phosphorylate the enzyme in the presence of Na^+ (ref. 9), and an analog of this compound containing a methylene bridge between ring and phosphorus, *p*-nitrobenzyl phosphonate (it is, therefore, incapable of phosphorylating the enzyme). First, kinetic studies were undertaken to demonstrate that the phosphonate analog could approach the active

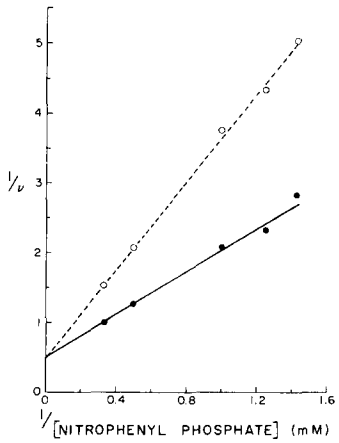

Fig. 4. Inhibition of K^+-dependent nitrophenyl phosphatase activity by nitrobenzyl phosphonate. The enzyme preparation was incubated in the standard medium (see Methods and Materials) but with the concentrations of nitrophenyl phosphate indicated, in the absence (●) or presence (○) of 3 mM nitrobenzyl phosphate. Enzyme velocity is expressed relative to that in the standard medium, defined as 1.0.

site: it was a potent competitive inhibitor toward the substrate (Fig. 4). Next, studies using Be^{2+} inactivation showed that, in the presence of Na^+ and nitrophenyl phosphate, the apparent affinity for K^+ was markedly altered (Fig. 5), just as with Na^+ and umbelliferone phosphate. But with the non-phosphorylating analog, *p*-nitrobenzyl phosphonate, even though it could attain the active site, no such change in affinity for K^+ was seen (Fig. 5).

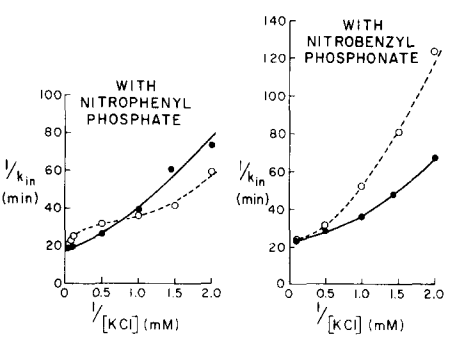

Fig. 5. Effects of nitrophenyl phosphate and nitrobenzyl phosphonate on inactivation by Be^{2+}. Inactivation of the enzyme was measured as in Fig. 3 in the absence (●) or presence (○) of 10 mM NaCl, with either 1.5 mM nitrophenyl phosphate (left-hand panel) or 1.5 mM nitrobenzyl phosphonate (right-hand panel). Data are presented as in Fig. 3.

CONCLUSIONS

A reasonable quantitative fit was obtained between K^+-dependent phosphatase activity in the presence of Na^+ and a model for phosphatase activity featuring alternative parallel enzymatic pathways. This model proposes that, in the presence

of Na^+ and a phosphorylating substrate, there is created an alternative enzymatic form permitting catalysis over the entire ($Na^+ + K^+$)-dependent ATPase pathway, in addition to the pathway over the terminal hydrolytic steps operative in the conventional phosphatase assays. The increase in apparent affinity for K^+ seen with Na^+ is thus due to the presence of this alternative pathway. Moreover, an alternative approach to measuring affinity for K^+ demonstrated that both Na^+ and substrate were necessary to increase affinity, whereas a non-phosphorylating analog of the substrate was ineffective, in accord with the model.

These considerations contrast with a model[10] for Na^+-induced stimulation based on two (or more) classes of K^+ sites. A good fit between model and data was also obtained, but no participation of substrate was proposed or investigated. However, the present data cannot disprove the possibility of there being more than one class of sites for K^+, merely they demonstrate that the available data do not require such a provision.

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