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INHIBITION OF THE ($Na^+ + K^+$)-DEPENDENT ATPase BY INORGANIC PHOSPHATE

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Summary

Inhibition of the ($Na^+ + K^+$)-dependent ATPase by inorganic phosphate, P_i, was examined in terms of product inhibition of the various activities catalyzed by an enzyme preparation from rat brain, and considered in terms of the specific transport processes of the membrane Na^+,K^+-pump that these activities reflect. The K^+-dependent phosphatase activity of the enzyme was most sensitive to P_i, and inhibition was competitive toward the substrate, nitrophenyl phosphate, as would be expected if P_i were released from the same enzyme form that bound substrate. However, this enzymatic activity does not seem to represent a transport process, and thus a cyclical discharge of K^+ may not be involved. The Na^+-dependent exchange activity was unaffected by P_i, in accord with the absence of P_i release in the reaction sequence. For the corresponding Na^+/Na^+ exchange function of the pump, which reportedly does not involve ATP hydrolysis either, prior release of P_i obviously cannot be required for Na^+ discharge. With the Na^+-dependent ATPase activity, measured using micromolar concentrations of ATP, P_i inhibited, but far less than with the phosphatase activity, and inhibition was not competitive toward ATP. Moreover, inhibition decreased as the Na^+ concentration was raised from 10 to 100 mM. This elevated concentration of Na^+ also led to substrate inhibition. For this ATPase activity, and the corresponding transport process, uncoupled Na^+ efflux, the findings suggest that Na^+ discharge follows P_i release, in contrast to Na^+/Na^+ exchange. The ($Na^+ + K^+$)-dependent ATPase activity, measured with millimolar concentrations of ATP and reflecting the coupled Na^+,K^+-transport function, was similarly sensitive to P_i, and again inhibition was not competitive toward ATP. However, in this case inhibition did not increase as the Na^+ concentration was lowered. For this activity, and the associated transport process, the site of Na^+ discharge in the overall reaction sequence remains unresolved.

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Introduction

The ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been linked convincingly to the Na^+,K^+-pump that in vivo effects the coupled transport of these ions across the plasma membrane [1–3]. In addition to this ATPase activity the enzyme also catalyzes several other reactions, and these may relate to alternative transport functions of the pump. In the absence of K^+ the enzyme can display Na^+-dependent ATPase activity, which seems to correspond to the uncoupled transport of Na^+ from the cell [3]. In the absence of K^+ the enzyme also can catalyze Na^+-dependent ADP/ATP exchange, and this activity reflects, in part, Na^+/Na^+ exchange across the membrane (further steps must, however, be involved in the transport process since it is sensitive to oligomycin whereas ADP/ATP exchange is not [3]). On the other hand, the relationship of a fourth reaction catalyzed by the enzyme, K^+-dependent phosphatase activity, to a transport role is doubtful [4], although the reaction does appear to correspond to the terminal hydrolytic steps of the overall ($Na^+ + K^+$)-dependent ATPase reaction [2,3].

For these reactions, and the transport functions, basic descriptions of the reaction processes are uncertain and the sequence of cation binding and discharge remains controversial [5,6]. Since the kinetic characteristics of product inhibition can afford information about reaction sequences [7], we have approached these issues by examining the effects on the separate enzymatic reactions of inorganic phosphate, P_i, since it would be expected to act as a product inhibitor toward the hydrolytic activities of the enzyme. The experiments described here indicate that P_i affects each of these reactions differently. These differences appear to correspond to variations in the reaction sequence which, in turn, may relate to the specific modes of cation transport that the enzymatic reactions reflect.

Methods and Materials

The enzyme preparation was obtained from rat brain microsomes by treatment with deoxycholate and then NaI, as previously described [8].

($Na^+ + K^+$)-dependent ATPase activity was measured in terms of the liberation of $^{32}P_i$ from incubations at 37°C with [γ-^{32}P]ATP. The standard incubation medium contained 30 mM histidine · HCl/Tris (pH 7.8), 90 mM NaCl, 10 mM KCl, 3.5 mM $MgCl_2$, and 3 mM ATP with tracer quantities of [γ-^{32}P]-ATP, plus the enzyme preparation. The reaction was stopped by the addition of trichloroacetic acid, to a final concentration of 5% (w/v), and the mixture was centrifuged. A portion of the supernatant material was diluted 7-fold with water, mixed with 50 mg of acid-washed charcoal, and centrifuged for 7 min at 10 000 × *g*. Radioactivity in the supernatant phase, $^{32}P_i$, was measured by liquid scintillation counting. Enzymatic activity in the absence of NaCl and KCl, which averaged only a few per cent of the ($Na^+ + K^+$)-dependent activity under these conditions, was measured concurrently, and was subtracted from the total activity in the presence of NaCl and KCl to give the ($Na^+ + K^+$)-dependent activity [8]. Because of variations in absolute activity between different enzyme preparations, velocities are expressed relative to the

$(Na^+ + K^+)$-dependent ATPase activity of a control incubation in the standard medium, defined as 1.0; the specific activity of the enzyme preparations averaged 2.6 μmol P_i liberated/min per mg protein.

Na^+-dependent ATPase activity was measured, and expressed, similarly. The standard medium contained 30 mM histidine · HCl · Tris (pH 7.8), 1 mM NaCl, 50 μM $MgCl_2$, and 9 μM ATP with tracer quantities of [γ-^{32}P]ATP, plus the enzyme preparation.

Na^+-dependent ADP/ATP exchange activity was measured, as previously described [9], in terms of the incorporation of ^{14}C into ATP from incubations at 37°C with unlabeled ATP and [U-^{14}C]ADP. The standard medium contained 30 mM histidine · HCl · Tris (pH 7.8), 20 mM NaCl, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM ADP with tracer quantities of [U-^{14}C]ADP, plus the enzyme preparation. Exchange activity in the absence of NaCl was measured concurrently, and subtracted from the total activity in the presence of NaCl to give the Na^+-dependent ADP/ATP exchange activity [9].

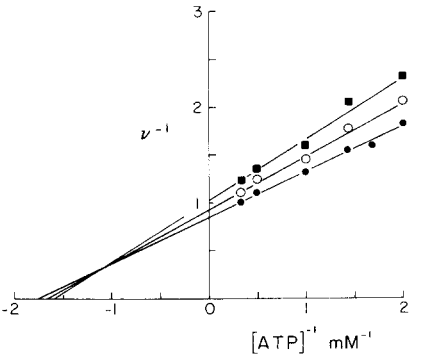
K^+-dependent phosphatase activity was measured in terms of the production of *p*-nitrophenol from incubations at 37°C with *p*-nitrophenyl phosphate, as described previously [10]. The standard medium contained 30 mM histidine · HCl · Tris (pH 7.8), 10 mM KCl, 3 mM $MgCl_2$, and 3 mM *p*-nitrophenyl phosphate, plus the enzyme preparation. Activity in the absence of KCl, which averaged only a few per cent of the K^+-dependent phosphatase activity under these conditions, was measured concurrently, and was subtracted from the total activity in the presence of KCl to give the K^+-dependent activity [10]. As with measurements of the ATPase activities, velocities are expressed relative to the K^+-dependent phosphatase activity of a control incubation in the standard medium defined as 1.0.

ATP, ADP, and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co., and converted to the Tris salts [8]. [γ-^{32}P]ATP was obtained from ICN, and [U-^{14}C]ADP from Amersham-Searle. Inorganic phosphate was added as the Tris salt, obtained by neutralization of orthophosphoric acid.

The data presented are averages of four or more experiments, each performed in duplicate or triplicate. The lines of the kinetic plots were fitted by eye. Values for the intercepts on the ordinates, summarized in Table I, were obtained by least-squares regression analysis.

Results

Inorganic phosphate inhibited the $(Na^+ + K^+)$-dependent ATPase activity when measured in the presence of near-saturating concentrations of ATP, Mg^{2+}, Na^+, and K^+, although the effect was slight: only 30% inhibition with 15 mM P_i (Fig. 1). Inhibition was somewhat greater at lower ATP concentrations, but P_i appeared to affect both V and K_m (Fig. 1). Since a true competitive inhibitor does not affect the intercept on the ordinate in double-reciprocal plots [7], the intercepts were evaluated by a least-squares linear regression analysis (Table I). Both 10 and 15 mM P_i increased the intercept (although the difference was statistically significant only at the higher concentration). Thus, in the terminology of Cleland [7], P_i is a noncompetitive inhibitor.



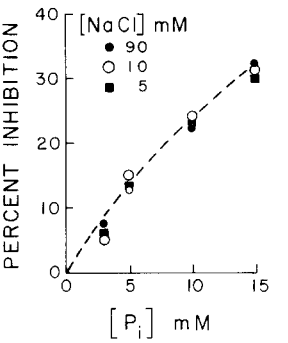


Fig. 1. Inhibition of $(Na^+ + K^+)$-dependent ATPase activity by P_i. The initial velocities of ATPase activity were estimated from incubations in media containing 30 mM histidine · HCl · Tris (pH 7.8), 90 mM NaCl, 10 mM KCl, the concentrations of ATP indicated, and $MgCl_2$ at concentrations 0.5 mM greater than that of ATP; experiments were performed in the absence of added P_i (•), and with 10 mM (○) and 15 mM (■) P_i as the Tris-salt. Data are presented in double-reciprocal form.

Fig. 2. Lack of effect of NaCl on inhibition of the $(Na^+ + K^+)$-dependent ATPase activity by P_i. Inhibition of ATPase activity was measured in the presence of the concentration of P_i indicated, from incubations in the standard medium adjusted to contain the concentration of NaCl shown. In these experiments there were no statistically significant differences in the extent of inhibition by P_i between any of the NaCl concentrations. For the different observations the S.E.M. ranged from 1 to 3% inhibition.

Reducing the concentration of NaCl in the incubation medium had no effect on inhibition by P_i (Fig. 2). Conversely, P_i did not affect the $K_{0.5}$ for either Na^+ or K^+ (data not presented).

When $(Na^+ + K^+)$-dependent ATPase activity was measured with 9 μM ATP, a concentration such that the high-affinity substrates sites are nearly saturated (K_m about 1 μM [11]) but the low-affinity substrate sites are not (K_m about 0.5 mM [11]), then inhibition by P_i was even less, and no effect of Na^+ or K^+

TABLE I

EFFECT OF INORGANIC PHOSPHATE ON THE INTERCEPT VALUES

The intercepts on the ordinate of double-reciprocal plots were calculated by least-squares linear regression analysis, from the individual experiments averaged in Figs. 1, 3 and 7; these values are presented, ±S.D.

Enzymatic activity	Concentration of added P_i (mM)	Intercept on the ordinate
$(Na^+ + K^+)$-dependent ATPase	0	0.85 ± 0.03
	10	0.93 ± 0.04
	15	1.04 ± 0.05 *
Na^+-dependent ATPase	0	0.75 ± 0.02
	5	0.92 ± 0.06 *
	10	1.10 ± 0.06 *
	15	1.30 ± 0.10 *
K^+-dependent phosphatase	0	0.43 ± 0.04
	1	0.39 ± 0.06
	2	0.45 ± 0.08
	3	0.64 ± 0.11

* Significantly different from control value ($P < 0.05$), as evaluated by Student's t-test.

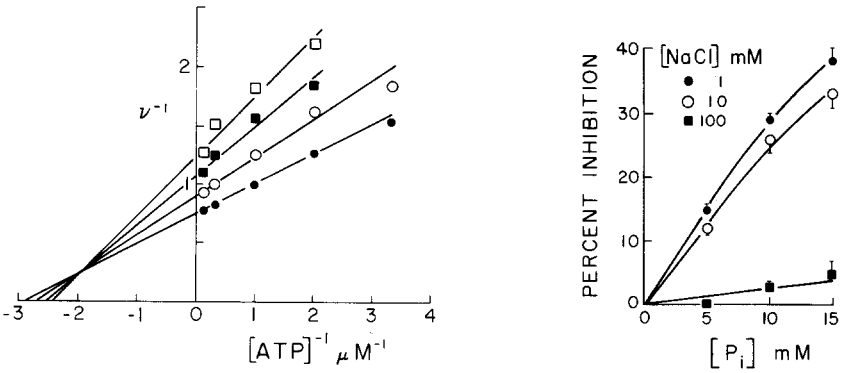


Fig. 3. Inhibition of Na^+-dependent ATPase activity by P_i. The initial velocities of ATPase activity were estimated from incubations in media containing 30 mM histidine · HCl · Tris (pH 7.8), 1 mM NaCl, 50 μM $MgCl_2$, and the concentrations of ATP indicated; experiments were performed in the absence of added P_i (●), and with 5 mM (○), 10 mM (■), and 15 mM (□) P_i. Data are presented as in Fig. 1.

Fig. 4. Effect of NaCl on inhibition of Na^+-dependent ATPase activity by P_i. The experiments were performed and the data are presented in Fig. 2, except that incubations were in the standard medium for Na^+-dependent ATPase activity, with modifications as indicated. The S.E.M. is indicated where it is larger than the data symbol; at all concentrations of P_i, inhibition was significantly less in media containing 100 mM NaCl. In the absence of P_i control values of the velocity were: with 1 mM NaCl (●), 1.0; with 10 mM NaCl (○), 1.03; and with 100 mM NaCl (■), 0.34.

concentration on the minimal inhibition could be discerned.

Na^+-dependent ATPase activity, measured in the absence of K^+ and also representing catalytic activity at the high-affinity substrate sites [11], was, however, more sensitive to P_i: 15 mM P_i inhibited 40% in the presence of 9 μM ATP. As in the case of ($Na^+ + K^+$)-dependent ATPase activity measured with ATP concentrations sufficient to fill both classes of substrates sites (Fig. 1), P_i appeared to affect both K_m and V (Fig. 3). Again, a regression analysis of the values for intercepts on the ordinate (Table I) indicates that P_i is a non-competitive inhibitor: values were increased by P_i, significantly in all cases (in these experiments the scatter of the data was somewhat greater, as reflected in the standard deviations, and this scatter is assumed to account for seeming deviations from linearity in the plots).

A notable difference between inhibition of the Na^+-dependent ATPase and ($Na^+ + K^+$)-dependent ATPase activities is the effect of NaCl: with the Na^+-dependent ATPase inhibition by P_i was almost completely abolished by 100 mM NaCl (Fig. 4). Under the experimental conditions used here to measure the Na^+-dependent ATPase activity, maximal velocity (in the absence of P_i) was achieved with 10 mM NaCl, and velocity declined when the NaCl was increased further. * Thus, not only did the percentage inhibition due to P_i

* Although several earlier studies [12—14] have described an increase in Na^+-dependent ATPase activity as the Na^+ concentration was raised from 10 to 100 mM, those measurements were made in the presence of millimolar $MgCl_2$ concentrations. Recent experiments (Flashner, M.S. and Robinson, J.D., unpublished observations) show that as the $MgCl_2$ concentration is raised from 50 to 2000 μM the apparent affinity for Na^+ in the low concentration range (0.5—5 mM) is decreased, and Na^+ in the high concentrations range (50—150 mM) then stimulates; this may be due to an effect of Mg^{2+} at the low-affinity substrate sites [15]. These observations are all from experiments in the absence of added P_i.

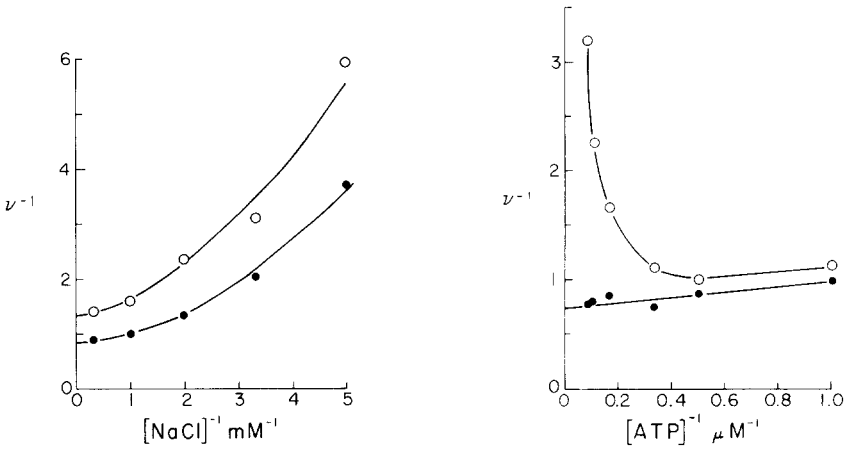


Fig. 5. Lack of effect of P_i on Na^+-activation of the Na^+-dependent ATPase activity. The initial velocities were estimated from incubations in the standard medium adjusted to contain the concentrations of NaCl indicated, in the absence of added P_i (●) or with 15 mM P_i (○). Data are presented as in Fig. 1. In both cases the $K_{0.5}$ for Na^+ is 0.4 mM.

Fig. 6. Effect of NaCl on substrate inhibition of the Na^+-dependent ATPase activity. Experiments were performed and the data are presented as in Fig. 3, except that the media contained either 1 mM (●) or 100 mM (○) NaCl. (No P_i was added in these experiments.)

decline as the NaCl concentration was raised (Fig. 4), but the percentage inhibition due to NaCl correspondingly declined as the P_i concentration was raised. On the other hand, when activation of the enzymatic activity by Na^+ was

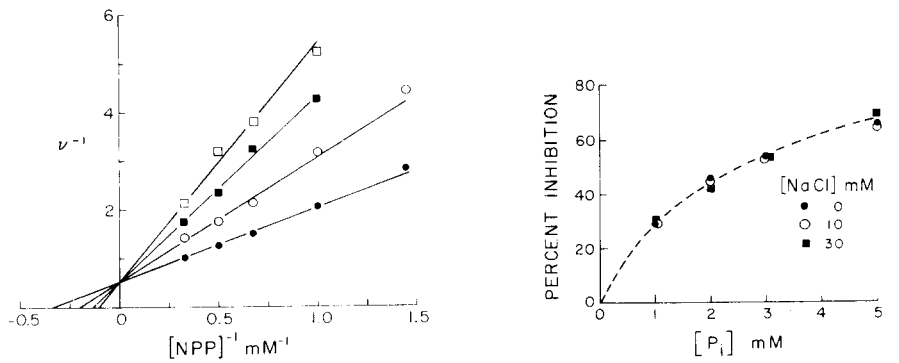


Fig. 7. Inhibition of K^+-dependent phosphatase activity by P_i. The initial velocities of phosphatase activity were estimated from incubations in media containing 30 mM histidine · HCl · Tris (pH 7.8), 10 mM KCl, 3 mM $MgCl_2$, and the concentrations of nitrophenyl phosphate (NPP) indicated; experiments were performed in the absence of added P_i (●), or with 1 mM (○), 2 mM (■), or 3 mM (□) P_i. Data are presented as in Fig. 1.

Fig. 8. Lack of effect of NaCl on inhibition of the K^+-dependent phosphatase activity by P_i. The experiments were performed and the data are presented as in Fig. 2, except that incubations were in the standard medium for the K^+-dependent phosphatase activity, with modifications as indicated. In these experiments there were no statistically significant differences in the extent of inhibition by P_i between experiments in the absence of NaCl and its presence. For the different observations the S.E.M. ranged from 1 to 5% inhibition.

examined at NaCl concentrations below 10 mM, P_i had no detectable effect on the $K_{0.5}$ for Na^+ (Fig. 5).

In the absence of P_i, inhibition of the Na^+-dependent ATPase by high concentrations of NaCl was accentuated at higher ATP concentrations, so that in the presence of 100 mM NaCl a double-reciprocal plot of velocity against ATP concentration showed marked substrate inhibition (Fig. 6).

Na^+-dependent ADP/ATP exchange activity catalyzed by this enzyme was not inhibited by 15 mM P_i, in the presence of NaCl concentrations from 2 to 20 mM (data not presented).

The remaining activity of the enzyme that was tested, K^+-dependent phosphatase activity, was, as shown previously [16], quite sensitive to inhibition by P_i: 3 mM P_i inhibited more than 50 per cent. Moreover, in this case P_i appeared to be a true competitive inhibitor (Fig. 7). A regression analysis of the values of the intercepts on the ordinate (Table I) showed no statistically significant difference between control values and those from experiments with three concentrations of P_i.

The phosphatase activity is measured in the absence of Na^+, and, indeed, Na^+ inhibits in the presence of optimal levels of K^+ [10]. P_i did not influence this inhibition by Na^+, nor did Na^+ affect inhibition by P_i (Fig. 8).

Discussion

Inorganic phosphate affected differently the four enzymatic reactions catalyzed by the ($Na^+ + K^+$)-dependent ATPase examined here. These results are considered in terms of product inhibition, by which the response of the initial velocity of a reaction to an added product reflects the reaction sequence [7]. For example, at the simplest level with an ordered release of products, the product that is released from the same enzyme state as that to which the (first) substrate binds (i.e., the product released last) will act as a true competitive inhibitor towards that substrate. Conversely, if other enzyme states intervene between release of a product and addition of substrate (e.g., release of a second product), then the first product will act as a noncompetitive inhibitor to the substrate. Thus, the different responses to P_i described here can suggest specific enzymatic and transport characteristics. These interpretations rest, however, on the assumption that P_i is inhibiting solely as a product of the reaction, which, though plausible, is not established exclusively.

(i) With the K^+-dependent phosphatase activity, P_i was a competitive inhibitor toward the substrate (Fig. 7), as previously shown [16]. This kinetic pattern is consistent with P_i being released from the same form of the enzyme as that to which the substrate binds [7]. Consequently, if K^+ is discharged with each reaction cycle, as would be expected with a transport process, K^+ must be discharged before P_i is released. It is, however, doubtful that this reaction can effect cation transport [4]. Nevertheless, the phosphatase reaction not only exhibits activating sites for K^+, with a $K_{0.5}$ near 2 mM, but also inhibitory low-affinity sites with a dissociation constant on the order of 100 mM [17], an affinity similar to that of the intracellular discharge sites for K^+ of the membrane Na^+,K^+-pump [18]. Thus the step(s) deficient for transport may involve the translocation of K^+ from acceptance to discharge sites.

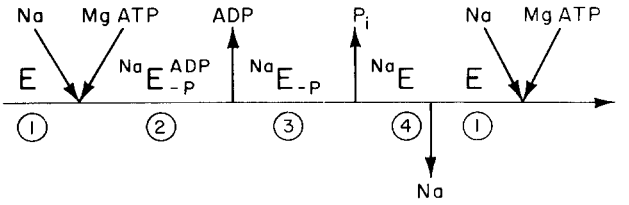


Fig. 9. Proposed reaction sequence for the Na^+-dependent ATPase activity. Na^+ and ATP bind to the free enzyme (E) (1), perhaps in random order, to form (2) the enzyme · phosphate complex [2,3], from which ADP subsequently dissociates (3). Next, phosphate is released from the enzyme · phosphate complex (4), followed sequentially by discharge of Na^+ (which in vivo would be from the opposite face of the membrane from that to which it bound). The cycle is repeated by subsequent addition of Na^+ and ATP to the free enzyme. Substrate inhibition occurring through ATP binding to an inactive form of the enzyme due to Na^+ at the discharge sites, dead-end inhibition, is not illustrated.

(ii) With the Na^+-dependent ADP/ATP exchange activity, in which no P_i is liberated, the addition of P_i, as would be expected, produced no inhibition. Here the enzymatic reaction resembles in part, Na^+/Na^+ exchange effected by the Na^+,K^+-pump, which appears also to involve a reversible transphosphorylation [3]. Garay and Garrahan [19] found no inhibition of Na^+/Na^+ exchange by P_i, and it is obvious that with this exchange the discharge of Na^+ cannot require a prior release of P_i.

(iii) The Na^+-dependent ATPase should represent the simplest case of active transport by the ATPase, the uncoupled Na^+ efflux [3,14], since only the high-affinity substrate sites are required [11,14] and Na^+ is the only monovalent cation involved in activation (enzyme) and transport (pump). With this ATPase activity, however, P_i was not a competitive inhibitor toward the substrate. Thus, if there is an ordered release of products from the enzyme, intervening step(s) are indicated between P_i release and ATP binding. This could occur if Na^+ were discharged after P_i is released, as shown in Fig. 9.

In this formulation, Na^+ released at its discharge sites is also a product. Under the standard assay conditions, with 1 mM NaCl, product inhibition by Na^+ would not be expected: although the $K_{0.5}$ for Na^+ at its activating sites is about 0.4 mM (Fig. 5), the dissociation constant at its discharge sites is on the order of 100 mM [20,21]. But if Na^+ were the last product released (Fig. 9), it should, at concentrations sufficient to occupy the discharge sites, act as a competitor toward ATP. This prediction, however, could not be verified because of the marked substrate inhibition occurring in the presence of high NaCl concentrations (Fig. 6). Nevertheless, this substrate inhibition can be accommodated by the formulation in Fig. 9 with the further stipulation that occupancy of the Na^+ discharge sites (occurring at high NaCl concentrations) stabilizes a dead-end form of the enzyme to which ATP also binds. As with all ad hoc stipulations, this explanation is not unique and requires further support.

An additional observation to be accommodated in a reaction scheme is the antagonism by high concentrations of NaCl toward the inhibition due to P_i (Fig. 4). It seems unlikely that this antagonsim results from Na^+, as a product, driving the reaction backward. If Na^+ normally were released after P_i (Fig. 9), then Na^+ should favor, by mass action, the preceding step(s) to which P_i binds, thereby increasing inhibition due to P_i; this is contrary to the observations

(Fig. 4). On the other hand, if P_i were released after Na^+, then adding P_i should favor, by mass action, the preceding steps to which Na^+ binds, thereby increasing inhibition due to Na^+; this too is contrary to the observations. It also seems unlikely that Na^+ exerts its antagonism toward P_i through occupancy of the activating sites for Na^+ (thereby pulling the reaction to the right by mass action) for the effect was most pronounced with 100 mM NaCl, a concentration at which the low-affinity discharge sites are being filled, not at 10 mM NaCl when the activating sites ($K_{0.5}$ near 0.4 mM) are nearly saturated. A fourth alternative to account for the observations is that in the presence of high concentrations of Na^+ a dead-end complex is formed, a complex to which P_i does not bind. This would represent, therefore, a step after P_i release (Fig. 9) and correspond to the dead-end complex involved above.

Post et al. [22] reported that phosphorylation of the enzyme with 20 μM ATP was inhibited by P_i, and that this inhibition was antagonized by 168 mM NaCl; similarly Cavieres and Glynn (quoted in ref. 14) found that 150 mM Na^+ prevented inhibition of Na^+-dependent ATPase activity (measured with 1 μM ATP) due to 2 mM P_i. These observations are in accord with the data and formulation presented here.

(iv) In studies on the ($Na^+ + K^+$)-dependent ATPase, Wade [23] recently reported that inhibition by P_i increased as the ATP concentration was lowered. On the other hand, Hexum et al. [24] previously found no effect of P_i on the K_m for ATP, and Garay and Garrahan [19], in studies on the Na^+,K^+-pump of erythrocytes, showed that the ATP concentration had no effect on inhibition by P_i. The data presented here (Fig. 1) support the interpretation of P_i acting as a noncompetitive inhibitor toward ATP, with little effect on the apparent K_m, unlike the inhibition of the K^+-dependent phosphatase and similar to that of the Na^+-dependent ATPase activity. But with the ($Na^+ + K^+$)-dependent ATPase activity the inhibition by P_i was not diminished as the NaCl concentration was raised (Fig. 2). This difference may reflect a more complex reaction process, which here involves binding and discharge of K^+ as well. Post et al. [22] have proposed that K^+ is discharged after the release of P_i, but whether in the functioning of coupled Na^+ and K^+ transport Na^+ is discharged before P_i release (in accord with the model for Na^+/Na^+ exchange) or after P_i release (in accord with the model for uncoupled Na^+ efflux pictured here) remains unresolved.

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