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Differential effects of substrates on three transport modes of the Na^+/K^+-ATPase

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With a purified Na^+/K^+-ATPase preparation reconstituted into phospholipid vesicles, Na^+/K^+, Na^+/Na^+, and uncoupled Na^+ transport were studied using three nucleotides and five substrates of the K^+-phosphatase reaction that this enzyme also catalyzes. For Na^+/K^+ exchange, CTP was half as effective as ATP and GTP one-twentieth; of the phosphatase substrates only carbamyl phosphate and 3-*O*-methylfluorescein phosphate produced significant transport and at merely 1% of the rate with ATP. For Na^+/Na^+ exchange, comparable rates of transport were produced by ATP, CTP, carbamyl phosphate and acetyl phosphate, although the actual rate of transport with ATP was only 2.4% of that for Na^+/K^+ exchange; slower rates occurred with GTP (69%), 3-*O*-methylfluorescein phosphate (51%), and nitrophenyl phosphate (33%). Only umbelliferone phosphate was ineffective. For uncoupled Na^+ transport results similar to those for Na^+/Na^+ exchange were obtained, but the actual rate of transport was still slower, 1.4% of that for Na^+/K^+ exchange. Thus, not only nucleotides but a variety of phosphatase substrates (which are phosphoric acid mixed anhydrides) can phosphorylate the enzyme at the high-affinity substrate site to form the E_1P intermediate of the reaction sequence. Oligomycin inhibited Na^+/K^+ exchange with ATP by half, but with carbamyl phosphate not at all; with CTP the inhibition was intermediate, one-fourth. By contrast, oligomycin inhibited Na^+/Na^+ exchange by one-fifth with all three substrates. A quantitative, steady-state kinetic model accounts for the relative magnitudes of Na^+/K^+ and Na^+/Na^+ exchanges with ATP, CTP, and carbamyl phosphate as substrates, as well as the extents of inhibition by oligomycin. The model requires that even when Na^+ substitutes for K^+ a slow step in the reaction sequence is the E_2 to E_1 conformational transition.

Introduction

Significant features of the Na^+/K^+-ATPase reaction sequence are: (i) Na^+-activated phosphorylation of the enzyme in the E_1 conformation, by ATP at the high-affinity substrate site, to form E_1P; (ii) a conformational change to E_2P, (iii) K^+-activated dephosphorylation to form E_2; and (iv) a conformational change back to E_1 (for reviews, see Refs. 1–3). The transitions between the two major conformational families (steps ii and iv) are associated with Na^+ and K^+ transport out of and into the cell, respectively, and in each case involves an intermediate form in which the bound cation is not readily exchangeable, i.e., it is occluded. The step between $E_2(K^+)$ and $E_1 + K^+$, from occluded K^+ to its discharge into the cytoplasm, is rate-limiting; however, this transition is markedly accelerated by ATP binding to the low-affinity site on $E_2(K^+)$ [4,5]. This reaction sequence corresponds to Na^+/K^+ exchange mediated by the enzyme. Two other transport modes are pertinent to the experiments described here. Na^+/Na^+ exchange reflects Na^+ occupying the extracellularly-accessible K^+ sites of E_2P, to activate dephosphorylation and be transported into the cell in lieu of K^+ [6,7]. An uncoupled Na^+ transport reflects dephosphorylation in the absence of either K^+ or Na^+ at the extracellularly-accessible K^+ sites, so that Na^+ transport out of the cell is unaccompanied by a corresponding transport inward [8]. (Distinct from the transport modes examined here is the ATP- and ADP-dependent Na^+/Na^+ exchange that

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Abbreviations: AcP, acetyl phosphate; CarbP, carbamyl phosphate; EDTA, ethylenediamine tetraacetate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate; Et_3N, triethylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NPP, nitrophenyl phosphate; 3-OMFP, 3-*O*-methylfluorescein phosphate; UmbP, umbelliferone phosphate.

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is associated with an ADP/ATP exchange reaction but not the consumption of ATP [1,2].)

With enzyme in broken membrane preparations the Na^+/K^+ transport mode is represented by Na^+/K^+-ATPase activity, while the other two transport modes are represented by Na^+-ATPase activity. For Na^+-ATPase activity there is no evidence of ATP occupying the low-affinity substrate site of E_2 to accelerate the conformational transition back to E_1 [8,9]. A further enzymatic reaction is also pertinent: K^+-phosphatase activity, in which various phosphoric acid mixed anhydrides (but not nucleoside triphosphates) are hydrolyzed by a K^+-activated process reflecting dephosphorylation of E_2P [1,2].

Although the enzyme catalyzes Na^+,K^+-activated hydrolysis of various nucleotides, only CTP, in addition to ATP, has been reported to energize cation transport, presumably Na^+/K^+ exchange, and at a rate comparable to that with ATP [10]; however, an early study using erythrocytes found only minimal transport with CTP [11]. And although the enzyme catalyzes K^+-activated hydrolysis of various acyl and phenolic anhydrides of phosphoric acid, only acetyl phospahte has been shown to energize cation transport [12,13]. Here are compared the abilities of nucleotides and of phosphatase substrates to serve as substrates for three modes of Na^+ transport: (i) to establish their ability to occupy the high-affinity substrate site and through that site to effect Na^+-activated phosphorylation of the enzyme, an obligatory initial step in the Na^+-transport scheme; (ii) to determine their efficacy at the low-affinity substrate site in accelerating the $E_2(K^+)$ to $E_1 + K^+$ transition; and (iii) to evaluate a specific steady-state kinetic model for Na^+/K^+ and Na^+/Na^+ exchange modes with diverse substrates, in the absence and presence of the inhibitor oligomycin.

Methods and Materials

The enzyme was prepared from medullae of frozen canine kidneys [14]; the specific activity was 15–20 μmol ATP hydrolyzed per min per mg protein at 37 °C.

The enzyme was reconstituted into vesicles following the procedure of Karlish and Stein [15]. In brief: the enzyme was solubilized (6 mg cholate/mg protein), mixed at 0 °C with soybean phospholipid vesicles (40 mg/mg protein) in the appropriate medium, frozen in liquid N_2, thawed, and sonicated for 1–2 min in a bath sonicator until partial clearing was obtained. The reconstitution medium contained 25 mM histidine (pH 7.0), 4 mM $MgCl_2$, 1 mM EDTA and either 140 mM KCl, NaCl, or the osmotically-equivalent amount of Tris-HCl (pH 7.0). After reconstitution the vesicles were centrifuged twice on Sephadex G-50 columns (equilibrated with 25 mM histidine, 1 mM EDTA, and 100 mM Tris-HCl (pH 7.0)) to remove extravesicular medium.

Transport was measured by mixing equal volumes of vesicle suspension and incubation medium to give final concentrations of 25 mM histidine (pH 7.0), 100 mM Tris-HCl (pH 7.0), 4 mM $MgCl_2$, 1 mM EDTA, and 30 mM NaCl containing about $5 \cdot 10^5$ dpm $^{22}Na^+$ per μmol NaCl. Osmolarity was adjusted to that in the vesicles with Tris-HCl. After incubation for 30–120 s at room temperature (21–23 °C) triplicate portions of each incubation were applied to Dowex 50 (Tris) columns to stop the reaction, and vesicles were eluted with ice-cold 0.35 M sucrose directly into vials for liquid scintillation counting. Substrate-dependent $^{22}Na^+$ uptake was determined by difference from triplicate samples in the absence and presence of substrate.

In experiments measuring inhibition by strophanthidin or oligomycin, the vesicles were preincubated with the inhibitor for 5 min at room temperature before adding the incubation medium; the specified concentration of inhibitor is that during preincubation.

Na^+/K^+-ATPase activity was measured in terms of P_i production following incubation at 37 °C in media containing 20 mM Hepes/Et_3N (pH 7.5), 0.1 mM EGTA, 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl [16]; Na^+-ATPase activity was measured similarly but with 100 mM NaCl and no KCl. The corresponding reactions with CTP and GTP were measured analogously. With CarbP as substrate P_i production was measured using the procedure of Wahler and Wollenberger [17]. With AcP as substrate the decrease in AcP content was measured by the hydroxamate method [18] using acetohydroxamic acid as a standard. Protein was measured by the procedure of Lowry et al. [19] using bovine serum albumin as a standard. Osmotic pressure was measured using a vapor pressure osmometer.

The steady-state equations were derived by the method of King and Altman [20].

Data are presented ± S.E., and statistical significance was evaluated using the *t*-test with a criterion of $P < 0.05$.

Frozen canine kidneys were obtained from Pel Freeze; ^{22}Na from New England Nuclear; oligomycin, strophanthidin, ATP, CTP, GTP, CarbP, AcP, 3-OMFP, and NPP from Sigma; and UmbP from ICN.

Results

$^{22}Na^+$ transport into K^+-containing vesicles

To compare the abilities of various substrates to energize the Na^+/K^+-exchange mode of the Na^+/K^+-ATPase, the purified enzyme was reconstituted into phospholipid vesicles containing 140 mM KCl. These vesicles were then incubated in media containing the substrate, 4 mM $MgCl_2$, and 30 mM $^{22}NaCl$ (with Tris-HCl added for osmotic balance). $^{22}Na^+$ transport into the vesicles thus represents Na^+ transport by en-

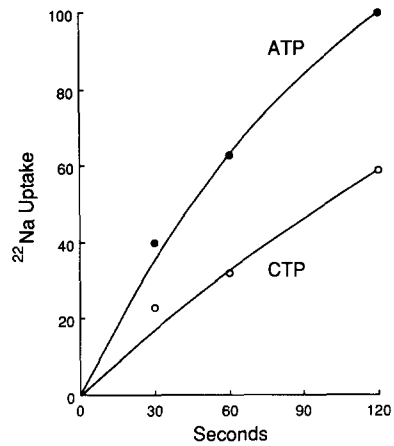

Fig. 1. Time course of $^{22}Na^+$ uptake into K^+ vesicles with ATP or CTP. $^{22}Na^+$ uptake into K^+ vesicles was measured after incubations for the specified times, with either no substrate, 3 mM ATP, or 3 mM CTP, as described under Methods and Materials. The substrate-dependent increment in $^{22}Na^+$ uptake, representing the average of two experiments each performed in triplicate, is plotted relative to that at 2 min with ATP, defined as 100 (this averaged 1.3 μmol $^{22}Na^+$ per mg protein per 2 min).

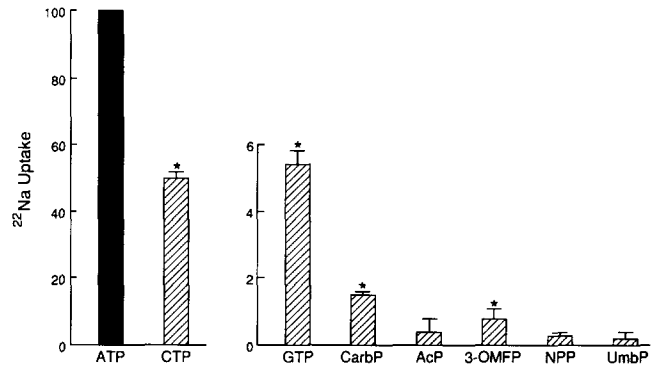

Fig. 2. Relative uptake of $^{22}Na^+$ into K^+ vesicles with various substrates. $^{22}Na^+$ uptake into K^+ vesicles was measured after incubation for 2 min with ATP, CTP, or GTP (3 mM), CarbP, AcP (5 mM), 3-OMFP (0.4 mM), NPP (6 mM), or UmbP (2.5 mM). Data are presented relative to that with ATP, defined as 100, as in Fig. 1. Note the different scales on the ordinates. The asterisks indicate an uptake statistically greater than that without substrate ($P < 0.05$).

zyme reconstituted inside-out: the transport is from the cytoplasmic face of the enzyme (exposed to the medium) to the extracellular face of the enzyme (exposed to the intravesicular contents). K^+ is presumably being transported in exchange for Na^+, in the opposite direction, although this flux was not measured.

With 3 mM ATP, $^{22}Na^+$ was transported into the vesicles in a time-dependent manner (Fig. 1). The ATP-dependent uptake during 2-min incubations averaged 1.3 μmol $^{22}Na^+$ per mg protein in the vesicles. Substituting 3 mM CTP for ATP decreased $^{22}Na^+$ transport to about half, at all the time points studied (Fig. 1).

With the other substrates $^{22}Na^+$ transport into the vesicles was far slower. Consequently, comparisons were made between values obtained from 2-min incubations, and the data are presented relative to the ATP-dependent transport, measured concurrently and defined as 100 (Fig. 2). $^{22}Na^+$ uptake significantly greater than that without added substrate occurred not only with the nucleotide GTP, but also with two substrates of the K^+-phosphatase reaction that the enzyme also catalyzes, CarbP and 3-OMFP. However, in all these cases the uptake was only a few percent of that with ATP (note difference in ordinate scale of Fig. 2). The other phosphatase substrates did not produce statistically significant uptake of $^{22}Na^+$.

$^{22}Na^+$ transport into Na^+-containing vesicles

The enzyme also catalyzes a Na^+/Na^+ exchange in which extracellular Na^+ substitutes for K^+ in activating enzyme dephosphorylation and is then transported from extracellular to cytoplasmic face of the enzyme. To compare the abilities of various substrates to energize this Na^+/Na^+ exchange, vesicles containing 140 mM NaCl were incubated in the same media as in the preceding experiments. Such $^{22}Na^+$ uptake when energized by CTP, CarbP, and AcP was not statistically different from that with ATP (Fig. 3), although ATP-dependent uptake into Na^+ vesicles was only a few percent of that into K^+ vesicles. With GTP, 3-OMFP, and NPP, $^{22}Na^+$ uptake was significantly less than with ATP, but still greater than that in the absence of substrate. Only UmbP was ineffective in energizing $^{22}Na^+$ uptake.

$^{22}Na^+$ transport into Tris-containing vesicles

A third transport mode of the Na^+/K^+-ATPase is uncoupled Na^+ efflux, in which Na^+ is transported from cytoplasmic to extracellular face of the enzyme without a corresponding transport of K^+ or Na^+ in the

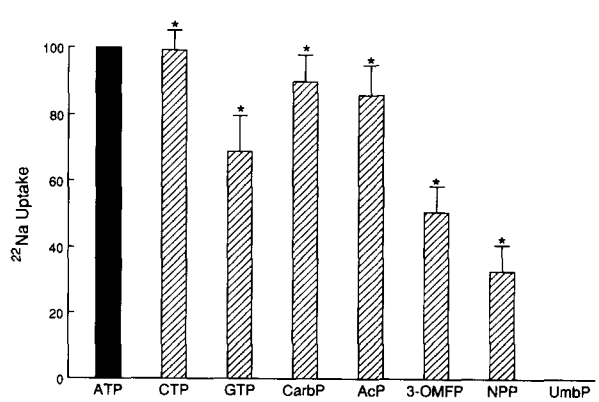

Fig. 3. Relative uptake of $^{22}Na^+$ into Na^+ vesicles with various substrates. $^{22}Na^+$ uptake into Na^+ vesicles was measured after incubation for 2 min with various substrates, and data are presented, as in Fig. 2 (with UmbP the value was 0). ATP-dependent uptake into Na^+ vesicles averaged only 2.4% of that into K^+ vesicles.

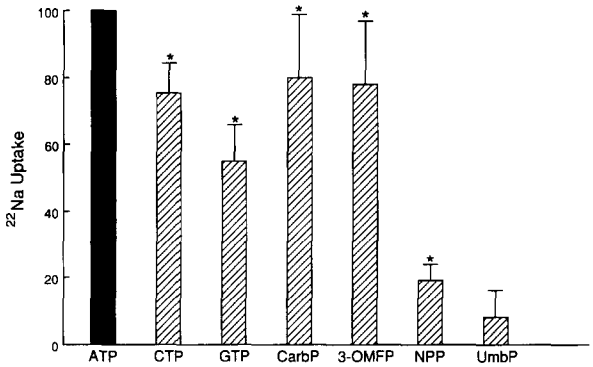

Fig. 4. Relative uptake of $^{22}Na^+$ into Tris vesicles with various substrates. $^{22}Na^+$ uptake into Tris vesicles was measured after incubation for 2 min with various substrates, and data are presented, as in Fig. 2. ATP-dependent uptake into Tris vesicles averaged only 1.4% of that into K^+ vesicles.

opposite direction. To compare the abilities of various substrates to energize this uncoupled flux, vesicles containing Tris were incubated in the same media as in the preceding experiments. As with $^{22}Na^+$ uptake into Na^+ vesicles, that into Tris vesicles was statistically significant with all the substrates tested except UmbP (Fig. 4). ATP-dependent uptake, however, was even slower than into Na^+ vesicles, averaging about 60% of that value. Moreover, $^{22}Na^+$ uptake was roughly the same with all the effective substrates except NPP, which again gave far lower values.

Effects of inhibitors on $^{22}Na^+$ transport

The cardioactive steroids are specific inhibitors of the Na^+/K^+-ATPase [1–3]. Because their binding site is on the extracellular face of the Na^+/K^+-ATPase, however, a lipid-soluble compound is necessary for these experiments in which transport is effected by enzyme oriented in the vesicles inside-out. Strophanthidin readily crosses cell membranes, and at a concentration of 10 μM it inhibited 85–100% of the $^{22}Na^+$ uptake into Na^+ vesicles energized by ATP, CTP, GTP, CarbP, AcP, and 3-OMFP (data not shown). However, although $^{22}Na^+$ uptake into Na^+-vesicles with NPP was significantly greater than in the absence of substrate (Fig. 3), 10 μM strophanthidin did not decrease that uptake significantly (data not shown).

Oligomycin inhibits the Na^+/K^+-ATPase, hindering the conversion of the E_1P to E_2P and the E_1 to E_2 conformations [21,22]. By contrast, oligomycin does not inhibit the K^+-phosphatase reaction as usually measured using NPP as substrate [23]. With K^+ vesicles, measuring Na^+/K^+ exchange, oligomycin inhibited significantly when ATP and CTP were substrates, but not with the nucleotide GTP nor the phosphatase substrate CarbP (Table I). On the other hand, with Na^+ vesicles, measuring Na^+/Na^+ exchange, oligomycin inhibited slightly with all four substrates, although the decreases in $^{22}Na^+$ uptake were not statistically significant (Table I).

TABLE I

Inhibition by oligomycin of Na^+/K^+ and Na^+/Na^+ exchanges

The experiments with K^+ vesicles and Na^+ vesicles were performed as described under Methods and Materials, with either 3 mM ATP, CTP, or GTP, or 5 mM CarbP, in the absence and presence of oligomycin (added to the vesicle suspension at a concentration of 10 μg/ml, for 5 min at room temperature, immediately before the transport incubation was begun.)

Substrate	Percent inhibition by oligomycin	
	Na^+/K^+ exchange	Na^+/Na^+ exchange
ATP	48± 2	21±12
CTP	26± 6	23±11
GTP	10±12	28±11
CarbP	0± 7	20±10

Activity of the enzyme in broken membranes

The Na^+/K^+-ATPase preparation isolated from kidney medulla consists of enzyme in broken membrane fragments, so that during standard assays both the cytoplasmic and extracellular faces of the enzyme are exposed to the medium. When substrate hydrolysis was

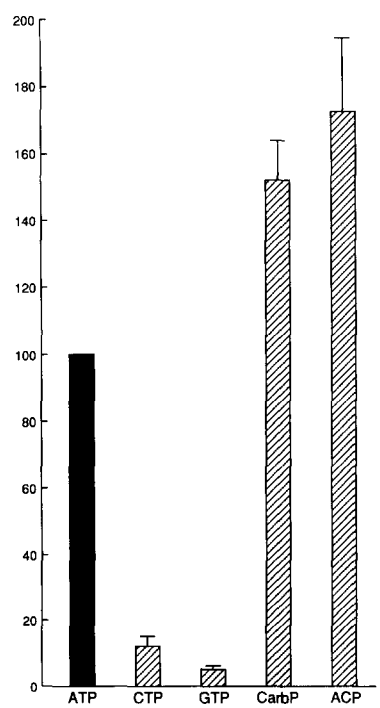

Fig. 5. Substrate hydrolysis in media containing 90 mM NaCl and 10 mM KCl. The Na^+/K^+-ATPase preparation, in a non-vesicular, broken-membrane form, was incubated at 37°C in media containing 90 mM NaCl and 10 mM KCl, and the rates of hydrolysis of 3 mM ATP, CTP, or GTP, or of 6 mM CarbP or AcP, were measured as described under Methods and Materials. These data are presented relative to that of ATP, defined as 100.

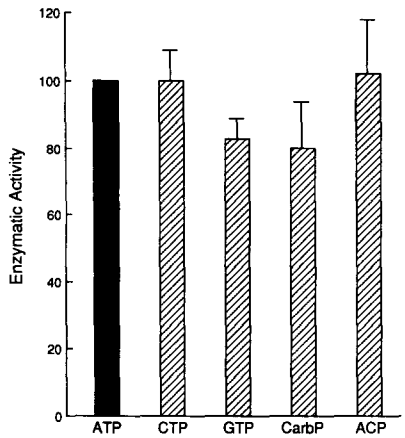

Fig. 6. Substrate hydrolysis in media containing 100 mM NaCl. The experiments were performed and the data are presented as in Fig. 5, except that the incubation media contained 100 mM NaCl and no KCl. ATP hydrolysis averaged only 3% of that with 90 mM NaCl and 10 mM KCl.

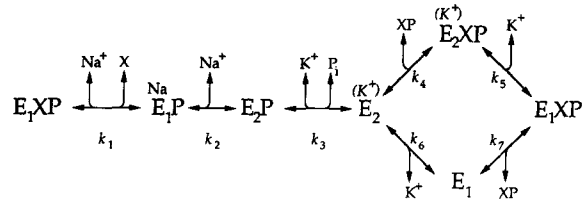

Fig. 7. Steady-state reaction scheme for Na^+/K^+ and Na^+/Na^+ exchanges. In this scheme XP stands for the substrate and X for the dephosphorylated substrate; in Na^+/Na^+ exchange, Na^+ at the extracellular face of the enzyme substitutes for K^+ (which is not present). All reactions are shown to be reversible; however, under initial velocity conditions where no product (X or P_i) is present, then reversal of the first and third steps cannot occur. Other details of the scheme are described in the text.

measured in media containing 90 mM NaCl and 10 mM KCl (near-optimal concentrations for the Na^+/K^+-ATPase reaction), Na^+/K^+-CTPase activity averaged 12% of Na^+/K^+-ATPase activity (Fig. 5), a far smaller fraction than when CTP energized Na^+/K^+ exchange (Figs. 1, 2); this discrepancy is probably due to the different accessibility of ligands in the two assays (see Discussion). A similar ratio of Na^+/K^+-CTPase to Na^+/K^+-ATPase activity was also found when the assay temperature was reduced to 20 °C (data not presented). By contrast, hydrolysis of the K^+-phosphatase substrates CarbP and AcP in 90 mM NaCl and 10 mM KCl was greater than Na^+/K^+-ATPase activity (Fig. 5), although Na^+/K^+ exchange energized by these substrates was only a few percent of that by ATP (Fig. 2).

When hydrolysis of these substrates was measured in media containing 100 mM NaCl, corresponding to conditions for measuring Na^+/Na^+ exchange, the activity was essentially the same with ATP, CTP, GTP, CarbP, and AcP (Fig. 6), as was Na^+/Na^+ exchange energized by these substrates (Fig. 3).

Steady-state kinetic model of Na^+/K^+ and Na^+/Na^+ transport modes

To define the differences among the substrates and to assess the adequacy of kinetic models of the enzyme

TABLE II

Parameters for steady-state kinetic model

The parameters, scaled to micromolar concentrations, were selected empirically; parameters changed to represent the effect of oligomycin are listed in parenthesis. Calculated relative velocities in the absence of oligomycin are: for Na^+/K^+ exchange with ATP, CTP, and CarbP, respectively, 100, 51, and 1.3; for Na^+/Na^+ exchange, 2.4 in all cases. Calculated percent inhibition by oligomycin is 52%, 29%, 0% for Na^+/K^+ exchange, respectively, and 15% for Na^+/Na^+ exchange in all cases.

Parameter	Na^+/K^+ exchange with			Na^+/Na^+ exchange with		
	ATP	CTP	CarbP	ATP	CTP	CarbP
k_1	1	1	1	1	1	1
k_2	3	3	3	3	3	3
	(0.195)	(0.195)	(0.195)	(0.195)	(0.195)	(0.195)
k_{-2}	2	2	2	2	2	2
k_3	12	12	12	0.3	0.3	0.3
k_4	2	2	2	0.2	0.2	0.2
k_{-4}	200	6000	4000	0.02	10	20
k_5	0.3	0.3	0.003	0.005	0.005	0.005
k_{-5}	0.03	0.5	0.015	0.002	0.002	0.002
	(0.002)	(0.032)	(0.001)	(0.00013)	(0.00013)	(0.0013)
k_6	0.002	0.002	0.002	0.005	0.005	0.005
k_{-6}	0.2	0.2	0.2	0.002	0.002	0.002
	(0.013)	(0.013)	(0.013)	(0.00013)	(0.00013)	(0.00013)
k_7	20	20	15	20	20	15
k_{-7}	2	1000	1500	2	1000	1500

reaction sequence, a simplified steady-state kinetic scheme was evaluated using plausible values for the various parameters (Fig. 7; Table II). For Na^+/K^+ exchange the first step represents Na^+ binding to enzyme with substrate (XP) at the high-affinity substrate site, enzyme phosphorylation by the substrate, and release of dephosphorylated substrate; under initial velocity conditions where no product is present this step is irreversible and thus no value for k_{-1} is listed (Table II). The second step involves the conformational change from E_1P to E_2P and the release of Na^+ from extracellularly-accessible sites; oligomycin is considered to inhibit the E_1P to E_2P transition. The third step includes K^+ binding to the extracellularly-accessible sites followed by K^+-activated dephosphorylation, and under initial velocity conditions it is irreversible. The fourth step depicts substrate binding to the low-affinity site of $E_2(K^+)$, and the fifth the conformational change to E_1K and release of K^+, with the substrate now bound to the high-affinity substrate site; oligomycin is considered to inhibit the reversal of the fifth step. An alternative pathway to these last two steps involves the conformational change from $E_2(K^+)$ to $E_1 + K^+$ in the absence of substrate at the low-affinity site, step 6, followed by substrate binding to the high-affinity site, step 7 (oligomycin is again considered to inhibit the reversal of the $E_2(K^+)$ to E_1 transition, step 6). Plausible values for the kinetic parameters with ATP, CTP, and CarbP as substrates give relative velocities for the Na^+/K^+ exchange of 100, 51, and 1.3 respectively (Table II), in excellent agreement with the observed ^{22}Na uptake (Fig. 2). For the Na^+/K^+ exchange, the differences among the substrates are accounted for chiefly by differences in k_{-4}, reflecting dissociation from the low affinity site, in k_5 and k_{-5}, reflecting the ability of the substrates to promote the $E_2(K)$ to $E_1 + K^+$ transition, and in k_{-7}, reflecting dissociation from the high-affinity substrate site.

For the Na^+/Na^+ exchange, extracellular Na^+ occupies the K^+ sites with: a consequent slower rate of dephosphorylation, represented by a smaller k_3; a slower rate of substrate binding, reflected in k_4; a shift in the poise of the E_2/E_1 transition reflected in k_5 and k_{-5}; and a diminished acceleration by nucleotides of the E_2 to E_1 transition and cation release, reflected in k_5. The shift in E_2/E_1 poise is consistent with K^+ favoring E_2 conformations and Na^+ favoring E_1 [24,25]. Calculated values for Na^+/Na^+ exchange give relative velocities of 2.4 for ATP, CTP, and CarbP (Table II), in excellent agreement with the observed ^{22}Na uptake (Fig. 3).

With oligomycin, k_2, k_{-5}, and k_{-6} are decreased to 6.5% of control values (Table II); the calculated inhibition with ATP, CTP, and CarbP is then 52%, 29%, and 0%, respectively, for the Na^+/K^+ exchange, and 15%, 15%, and 15% for the Na^+/Na^+ exchange, again in excellent agreement with the observed values (Table I).

To determine how dependent the calculated values are on the values assigned to the kinetic parameters, each was doubled in turn while the others were held constant. For Na^+/K^+ exchange with ATP, the only parameters that when doubled altered the velocity more than 10% were k_1 (increased velocity by 12%) and k_5 (increased velocity by 55%); similarly, for Na^+/Na^+ exchange with ATP, only doubling k_5 affected velocity more than 10% (in this case increasing it by 94%).

Discussion

These observations relate to three major issues: the ability of various substrates to occupy the high-affinity substrate site and there phosphorylate the enzyme; the ability of these substrates to occupy the low-affinity substrate-site and there accelerate the E_2 to E_1 transition; and the mechanism by which oligomycin can inhibit differently with various substrates and alternative transport modes.

(i) The ability of the substrates to energize Na^+ transport indicates that they can occupy the high-affinity substrate site of E_1 and there phosphorylate the enzyme to form E_1P. This is true not only of the nucleotides ATP, CTP, and GTP, but also of the phosphoric acid anhydride phosphatase substrates: both the carboxylic (acyl) phosphates, CarbP and AcP, and at least one phenolic phosphate, 3-OMFP (and perhaps also NPP). Since E_1P is a 'high-energy' intermediate, able to transfer its phosphate to ADP to form ATP, the efficacy of the phosphatase substrates confirms their classification as 'high-energy' compounds. Where enzyme dephosphorylation is slow, as in Na^+/Na^+ exchange and uncoupled Na^+ transport, the differences in rates of $^{22}Na^+$ uptake probably reflect differences in rates of enzyme phosphorylation, and the inability to detect $^{22}Na^+$ uptake with UmbP as substrate may represent an even slower rate of phosphorylation by this phenolic phosphate. Indeed, the rank order of the phosphatase substrates in terms of $^{22}Na^+$ uptake parallels their order in terms of K^+-phosphatase activity: CarbP $\geq$ AcP > 3-OMFP > NPP > UmbP (Robinson, J.D., unpublished observations).

In the K^+-phosphatase reaction, which is catalyzed by the E_2 conformation [9,26,27], hydrolysis of AcP involves enzyme phosphorylation whereas hydrolysis of NPP does not [28]; indirect evidence suggests that hydrolysis of 3-OMF also involves enzyme phosphorylation [29]. Why the phosphorylating pathway of the phosphatase reaction is limited to certain substrates and why that pathway is faster is not apparent.

(ii) In Na^+/K^+ exchange, where the $E_2(K^+)$ to $E_1 + K^+$ transition is rate limiting, the process is about half as fast when CTP is substituted for ATP and one-twentieth as fast when GTP is substituted (Fig. 2). These data are in accord with earlier studies on en-

zymatic processes in which the rank order for accelerating that transition is ATP > CTP > GTP [30,31].

There is, however, a marked discrepancy between the ratio of Na^+/K^+ exchange mediated by CTP vs. ATP, for which CTP is about half as effective as ATP (Figs. 1, 2), and the ratio of Na^+/K^+-CTPase vs. ATPase activity, for which CTP is only one-eighth as effective (Fig. 5). The most obvious difference between the assay conditions is the segregation of ions in the transport experiments, where only the extracellular sites are exposed to K^+ and only the cytoplasmic sites to Na^+. The relatively slower velocity with CTP in the broken-membrane nucleotidase experiments thus may reflect the presence in those experiments of K^+ acting on cytoplasmic sites: K^+ at the cytoplasmic face of the enzyme may further impede de-occlusion, the step where CTP is notably inferior to ATP.

Na^+/K^+ exchange energized by the phosphatase substrates is even slower than with GTP. This does not represent a generally slower rate of E_1P formation with the compounds, since CarbP and AcP are at least as effective as GTP in Na^+/Na^+ exchange. Instead, the slower rate of Na^+/K^+ exchange is attributable to the phosphatase substrates being unable to occupy the adenine-binding region of the low-affinity substrate site. Common formulations for the enzymatic reaction sequence depict distortion of the substrate site occurring with the E_1 to E_2 transition, producing the decrease in affinity for ATP [32,33]. Also accompanying this distortion is an apparent separation of the adenine-binding site from the phosphate-accepting site [34,35]; the latter is involved in K^+-phosphatase activity with AcP [28] and perhaps with other phosphatase substrates as well. However, 3-OMFP apparently binds to the adenine-binding region of the low-affinity site through its fluorescein ring system, accounting for its low K_m in the phosphatase reaction [35], yet Na^+/K^+ exchange with 3-OMFP is no faster than that with CarbP, which would not be expected to bind at the adenine site. This failure by 3-OMFP may represent an inadequate spacing between the fluorescein and phosphate portions, which is, in fact, quite different from that in the nucleotides.

On the other hand, since K^+-phosphatase activity with CarbP and AcP is faster than Na^+/K^+-ATPase activity, the rapid $(Na^+ + K^+)$-stimulated hydrolysis of CarbP and AcP (compared to ATP) and the slow Na^+/K^+ exchange energized by these substrates probably reflect the additional occurrence in the hydrolysis assays of K^+-phosphatase activity. K^+-phosphase activity is not associated with transport, and Na^+ acts as a relatively weak competitor toward K^+ [1,2].

(iii) Oligomycin inhibits the transition of E_1P to E_2P and of E_1 to E_2 [21,22], yet oligomycin has quite different effects on Na^+/K^+ and Na^+/Na^+ exchanges and on transport energized by various substrates. A quantitative, steady state model of a simplified reaction scheme can accommodate these observations (Fig. 7, Table II); moreover, the model also suggests properties of the reaction sequence that may not have seemed obvious.

For Na^+/K^+ exchange, the usual considerations can account for ATP accelerating the reaction by increasing the rate of step 5, with CTP accelerating less by increasing the rate of that step less. With CarbP occupying only the phosphatase portion of the low-affinity site, and thereby increasing the rate of that step little more than in its absence, the overall rate of Na^+/K^+ exchange is much slower than with ATP or CTP. And, although oligomycin inhibits the same forward step with all three substrates, represented by the decrease in k_2, the consequent variation in inhibition (from 0 to 50%) seems straightforward: with CarbP late steps in the sequence are so slow that inhibition of an earlier step has no effect, whereas with ATP the late steps (following the more rapid, upper pathway) are fast enough for the 15-fold slowing of k_2 to produce a 2-fold slowing overall.

For Na^+/Na^+ exchange the model depicts a much slower rate of dephosphorylation (k_3), representing the lesser efficacy of Na^+ in activating that step compared to K^+. It also depicts no effect of any substrate on accelerating the E_2 to E_1 transition, in accord with the lack of stimulation of Na^+-ATPase activity by ATP at concentrations expected to fill the low-affinity substrate site [8,9]: either because substituting Na^+ for K^+ on E_2 alters further the substrate site so that ATP cannot bind, or, more likely, because ATP binding to the low-affinity site does not affect the transition when Na^+ rather than K^+ is bound to E_2.

The rates assigned to the latter steps, however, are constrained by the observation that oligomycin inhibits Na^+/Na^+ exchange relatively little. It might seem that a slower rate of dephosphorylation (k_3) would serve an equalizing role in making oligomycin inhibit Na^+/Na^+ exchange comparably with all substrates, and to a minimal extent, just as having both k_5 and k_6 small resulted in minimal inhibition of the Na^+/K^+ exchange when CarbP is the substrate. Nevertheless, although it may not seem intuitively obvious, numerical evaluation of the model reveals that low levels of inhibition by oligomycin can occur only when steps subsequent to dephosphorylation are slower than dephosphorylation. (Expanding the model, to insert additional steps between the oligomycin-sensitive E_1P to E_2P transition and the dephosphorylation, to depict Na^+ release and K^+ binding, still does not remove this stricture.) Thus, the effects of oligomycin as incorporated in the model of Na^+-accelerated ephosphorylation, imply that the transition from E_2 to E_1 is rate limiting even with Na^+, although not to such an extent as with K^+. If this slow step involves occlusion of Na^+ in this limb of the

reaction sequence, then it also differs from occlusion of K^+ in its insensitivity to de-occlusion by ATP.

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References

1 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–179.
2 Glynn, I.M. (1985) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 3, pp. 35–114, Plenum Press, New York.
3 Jørgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.
4 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.
5 Beaugé, L.A. and Glynn, I.M. (1979) Nature 280, 510–512.
6 Lee, K.H. and Blostein, R. (1980) Nature 285, 338–339.
7 Forgac, M. and Chin, G. (1982) J. Biol. Chem. 257, 5652–5655.
8 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. (Lond.) 256, 465–496.
9 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006–1019.
10 Svinukhova, I.A. and Boldyrev, A.A. (1987) FEBS Lett. 214, 335–338.
11 Glynn, I.M. and Hoffman, J.F. (1971) J. Physiol. (Lond.) 218, 239–256.
12 Beaugé, L. and Berberian, G. (1984) Biochim. Biophys. Acta 772, 411–414.
13 Campos, M., Berberian, G. and Beaugé, L. (1988) Biochim. Biophys. Acta 938, 7–16.
14 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
15 Karlish, S.J.D. and Stein, W.D. (1982) J. Physiol. (Lond.) 328, 295–316.
16 Robinson, J.D. (1967) Biochemistry 6, 3250–3258.
17 Wahler, B.E. and Wollenberger, A. (1958) Biochem. Z. 329, 508–520.
18 Lipman, F. and Tuttle, L.C. (1945) J. Biol. Chem. 159, 21–28.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 King, E.L. and Altman, C. (1956) J. Phys. Chem. 60, 1375–1378.
21 Fahn, S., Koval, G.J. and Albers, R.W. (1966) J. Biol. Chem. 241, 1882–1889.
22 Kalrish, S.J.D., Yates, D.W. and Glynn, I.M. (1978) Biochim. Biophys. Acta 525, 252–264.
23 Robinson, J.D. (1971) Mol. Pharmacol. 7, 238–246.
24 Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399–415.
25 Karlish, S.J.D. and Yates, D.W. (1978) Biochim. Biophys. Acta 527, 115–130.
26 Robinson, J.D., Robinson, L.J. and Martin, N.J. (1984) Biochim. Biophys. Acta 772, 295–306.
27 Berberian, G. and Beaugé, L. (1985) Biochim. Biophys. Acta 821, 17–29.
28 Shaffer, E., Azari, J. and Dahms, A.S. (1978) J. Biol. Chem. 253, 5696–5706.
29 Davis, R.L. and Robinson, J.D. (1988) J. Bioenerg. Biomembr. 20, 571–584.
30 Robinson, J.D. (1982) Arch. Biochem. Biophys. 213, 650–657.
31 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., Zhao, R.S. and De Pont, J.J.H.H.M. (1986) Biochim. Biophys. Acta 861, 259–266.
32 Smith, R.L., Zinn, K. and Cantley, L.C. (1980) J. Biol. Chem. 255, 9852–9859.
33 Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2357–2366.
34 Robinson, J.D., Leach, C.A. and Robinson, L.J. (1986) Biochim. Biophys. Acta 872, 294–304.
35 Davis, R.L. and Robinson, J.D. (1988) Biochim. Biophys. Acta 953, 26–36.