Biochimica et Biophysica Acta, 772 (1984) 295–306
Elsevier

BBA 72093

EFFECTS OF OLIGOMYCIN AND QUERCETIN ON THE HYDROLYTIC ACTIVITIES OF THE $(Na^+ + K^+)$-DEPENDENT ATPase

JOSEPH D. ROBINSON, LISA J. ROBINSON and NANCY J. MARTIN

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

(Received January 31st, 1984)

Key words: Oligomycin; Quercetin; $(Na^+ + K^+)$-ATPase; (Dog kidney)

Quercetin inhibited a dog kidney $(Na^+ + K^+)$-ATPase preparation without affecting K_m for ATP or $K_{0.5}$ for cation activators, attributable to the slowly-reversible nature of its inhibition. Dimethyl sulfoxide, a selector of E_2 enzyme conformations, blocked this inhibition, while the K^+-phosphatase activity was at least as sensitive to quercetin as the $(Na^+ + K^+)$-ATPase activity, all consistent with quercetin favoring E_1 conformations of the enzyme. Oligomycin, a rapidly-reversible inhibitor, decreased the K_m for ATP and the $K_{0.5}$ for cation activators, and its inhibition was also diminished by dimethyl sulfoxide. Although oligomycin did not inhibit the K^+-phosphatase activity under standard assay conditions, a reaction presumably catalyzed by E_2 conformations, its effects are nevertheless accommodated by a quantitative model for that reaction depicting oligomycin as favoring E_1 conformations. The model also accounts quantitatively for effects of both dimethyl sulfoxide and oligomycin on V_{max}, K_m for substrate, and $K_{0.5}$ for K^+, as well as for stimulation of phosphatase activity by both these reagents at low K^+ but high Na^+ concentrations.

Introduction

The conventional reaction sequence of the $(Na^+ + K^+)$-ATPase depicts a cycle between two major conformational states, E_1 and E_2, each existing in phosphorylated and dephosphorylated forms [1–3]:

$$E_1 \rightarrow E_1 - P \rightarrow E_2 - P \rightarrow E_2 \rightarrow E_1$$

Early studies by Albers and associates [4] established that oligomycin inhibited the transition from $E_1 - P$ to $E_2 - P$, and subsequent experiments demonstrated a corresponding slowing of the conversion of E_1 to E_2 [5–7]. Nevertheless, oligomycin did not inhibit phosphorylation by P_i to form $E_2 - P$ [8], nor did it seem to inhibit the K^+-phosphatase activity of the enzyme [9–11] even though this reaction appears to be catalyzed by a K-bound E_2 state, related through the following equilibria [12]:

$$E_2 \rightleftharpoons E_1 + K \rightleftharpoons E_1K \rightleftharpoons E_2K$$

On the other hand, Kuriki and Racker [13] found that quercetin, which similarly hindered the conversion of $E_1 - P$ to $E_2 - P$, did inhibit both K^+-phosphatase activity and enzyme phosphorylation by P_i.

The experiments described here arose from considerations of the discrepant effects of these two inhibitors having presumably similar actions: shifting equilibria between enzyme conformations toward E_1 states. With quercetin, measurements of both $(Na^+ + K^+)$-ATPase and K^+-phosphatase activities were generally consistent with such shifts in conformational equilibria; however, since

Abbreviations: K^+-phosphatase, K^+-dependent *p*-nitrophenyl phosphatase; Me_2SO, dimethyl sulfoxide; $(Na^+ + K^+)$-ATPase, $(Na^+ + K^+)$-dependent ATPase.

quercetin acted as a slowly-reversible inhibitor, the competition toward reagents favoring E_2 states, otherwise expected, was not apparent. With oligomycin, a rapidly-reversible inhibitor, the minimal inhibition of K^+-phosphatase activity could still be accommodated by an expanded version of a reaction model recently proposed [12], despite the dependence of such activity on E_2 states: although oligomycin induced a many-fold shift in the equilibrium from E_2K toward E_1K states, the $K_{0.5}$ for K^+ was altered only slightly.

These experiments thus also provided the opportunity to test, with a reagent favoring E_1 states, the scheme for the K^+-phosphatase reaction previously examined in terms of a reagent thought to favor E_2 states, Me_2SO. The extended model presented here, which incorporates substrate interactions as well as effects of Na^+ and K^+, fits the data quantitatively over a broad range of experimental perturbations. With this model both inhibition and stimulation due to oligomycin as well as stimulation due to Me_2SO are attributable to shifts in equilibria between E_1 and E_2 states. Nevertheless, although the chosen reaction parameters can account for stimulation of the phosphatase activity by oligomycin in the presence of Na^+ and K^+, the mechanism by which oligomycin might alter the specific parameter underlying the calculated stimulation is uncertain.

Methods and Materials

The enzyme preparations was obtained from medullae of frozen canine kidneys, following the procedure of Jørgensen [14]. The specific activity of the $(Na^+ + K^+)$-ATPase activity ranged from 16 to 22 μmol P_i liberated/mg protein per min at 37°C.

$(Na^+ + K^+)$-ATPase activity was measured at 37°C in terms of the production of P_i, as described previously [15]. The standard incubation medium contained 30 mM histidine · HCl/Tris (pH 7.8), 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl. The enzyme was routinely preincubated in this medium but without the ATP for 8 min at 37°C, either with 2 μl of ethanol per ml of medium (control) or with 2 μl of an ethanolic solution of oligomycin or quercetin; the incubation was then initiated by adding the ATP. Na^+-ATPase activity was measured similarly, except that KCl was omitted and the NaCl concentration was 100 mM. $(Na^+ + K^+)$-CTPase and Na^+-CTPase activities were measured by substituting 3 mM CTP for ATP in the corresponding media. K^+-Phosphatase activity was measured in terms of nitrophenol production from incubations at 37°C with nitrophenyl phosphate as substrate [16]. The standard incubation medium contained 30 mM histidine · HCl/Tris (pH 7.8), 3 mM nitrophenyl phosphate, 3 mM $MgCl_2$, and 10 mM KCl. Preincubation with ethanol or ethanolic solutions of oligomycin or quercetin was in the absence of nitrophenyl phosphate, in analogy with the ATPase assays, and the incubation was initiated by addition of substrate.

Data presented are averages of four or more experiments, each performed in duplicate to quadruplicate, with the standard error of the mean where appropriate. In the figures the solid lines are fitted by eye; the broken lines (dashed or dotted) are calculated from the designated formulae and parameters.

Frozen dog kidneys were obtained from Pel-Freeze; and ATP, ADP, CTP, *p*-nitrophenyl phosphate, oligomycin, and quercetin from Sigma.

Results

ATPase and CTPase activities

Both oligomycin and quercetin inhibited $(Na^+ + K^+)$-ATPase activity in a dose-dependent fashion. With 10 μg/ml oligomycin maximal inhibition occurred during a 2-min preincubation before ATP was added to initiate the assay incubation; however, with 2 μg/ml quercetin inhibition increased with preincubation time for up to 5 or 6 min. Consequently, to assure full interaction and to facilitate comparisons of effects, both drugs were routinely preincubated with the enzyme for 8 min before assay (Table I). Following such preincubation with 10 μg/ml oligomycin for 8 min, a 10-fold dilution accompanying addition of ATP brought the extent of inhibition to that caused by 1 μg/ml. On the other hand, following preincubation with 2 μg/ml quercetin, a 10-fold dilution restored activity from 51% of control activity only to 74%, whereas preincubation with 0.2 μg/ml quercetin reduced $(Na^+ + K^+)$-ATPase activity

only slightly, to 93% of control activity. Thus, in comparison to oligomycin, both the onset of inhibition by quercetin and its reversal by dilution were distinguishably slower. Inhibition by quercetin could, however, be removed by a 50-fold dilution, centrifugation, and resuspension of the enzyme.

The effects of these two inhibitors on the kinetic parameters of the $(Na^+ + K^+)$-ATPase activity were also readily distinguishable. Oligomycin sharply decreased the K_m for ATP, to less than half the control value, whereas quercetin had relatively little effect (Fig. 1).

Similarly, oligomycin reduced the $K_{0.5}$ for KCl as activator of the $(Na^+ + K^+)$-ATPase activity from 0.8 mM to 0.4 mM (Fig. 2) and for NaCl as activator from 8 mM to 3 mM (Fig. 3). Again, quercetin had little effect on either parameter.

ADP behaves as a competitive inhibitor toward ATP [17,18], although it may act not only through competing with ATP for the substrate sites but also by depleting $E_1 - P$ through transphosphorylation to form ATP [4]. In the presence of 2 mM ADP and 1 mM ATP, oligomycin inhibited slightly more than with 3 mM ATP, but quercetin inhibited less (Table I).

Me_2SO decreases $(Na^+ + K^+)$-ATPase activity, presumably by favoring E_2 conformational states [12], and thus might be expected to antagonize

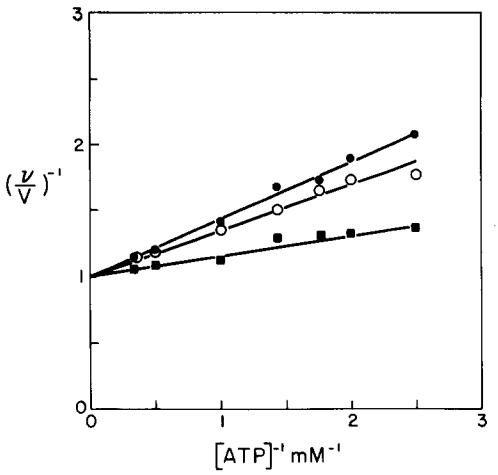


Fig. 1. Effects of oligomycin and quercetin on substrate dependence. (Na+K)-ATPase activity was measured in the standard medium (see Methods) modified to contain the concentrations of ATP shown (with the $MgCl_2$ concentration 0.5 mM higher throughout). Velocities are plotted in double-reciprocal form relative to the maximal velocity, from experiments in the absence of inhibitors (●), or with oligomycin, 10 μg/ml (■), or quercetin, 2 μg/ml (○).

inhibition caused by reagents favoring E_1 states. At a concentration of Me_2SO that reduced activity 29%, oligomycin inhibited only about half as much as in the absence of Me_2SO, and quercetin scarcely inhibited at all (Table I).

In the absence of K^+ the enzyme can catalyze a Na^+-ATPase activity with a maximal velocity about one-tenth that of the $(Na^+ + K^+)$-ATPase [18]. Oligomycin inhibited the Na^+-ATPase slightly less than the $(Na^+ + K^+)$-ATPase activity, although quercetin inhibited both equally (Table I).

Substituting CTP for ATP reduces the $(Na^+ + K^+)$-dependent activity by three-fourths [19], but oligomycin then inhibited appreciably less than in the case of the $(Na^+ + K^+)$-ATPase, whereas quercetin inhibited the $(Na^+ + K^+)$-CTPase markedly more (Table I). Relative effects on the Na^+-CTPase activity were analogous to those on the Na^+-ATPase activity: oligomycin inhibited somewhat less than with the $(Na^+ + K^+)$-CTPase activity, whereas quercetin inhibited both activities about the same.

TABLE I

INHIBITION OF NUCLEOTIDE TRIPHOSPHATASE ACTIVITIES

$(Na^+ + K^+)$-ATPase, Na^+-ATPase, $(Na^+ + K^+)$-CTPase, and Na^+-CTPase activities were measured in the standard media (see Methods), modified by the additions noted, in the absence and presence of oligomycin or quercetin. Percentage inhibition is calculated relative to the corresponding control, treated identically except for the absence of inhibitor.

Activity and additions	Percent inhibition	
	with oligomycin (10 μg/ml)	with quercetin (2 μg/ml)
$(Na^+ + K^+)$-ATPase	63±2	49±2
plus ADP, 2 mM	74±2	33±3
plus Me_2SO, 100 μl/ml	38±1	6±2
Na^+-ATPase	53±3	50±3
$(Na^+ + K^+)$-CTPase	22±4	95±3
Na^+-CTPase	12±3	81±4

K^+-Phosphatase activity

With the K^+-phosphatase activity catalyzed by

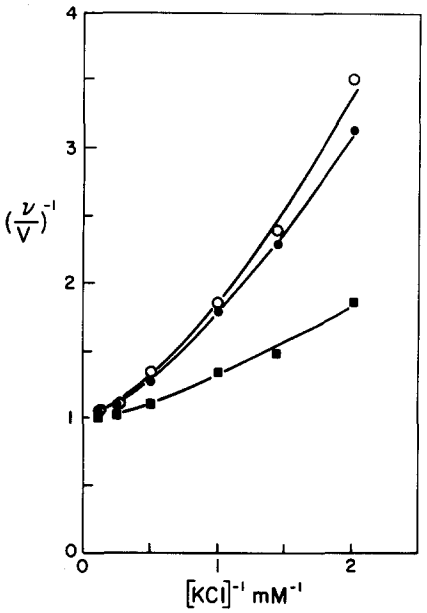


Fig. 2. Effects on K^+-activation. Experiments were performed and data are presented as in Fig. 1, except that the ATP concentration was 3 mM whereas the concentrations of KCl were as shown: in the absence of inhibitors (●), or with oligomycin, 10 μg/ml (■), or quercetin, 2 μg/ml (○).

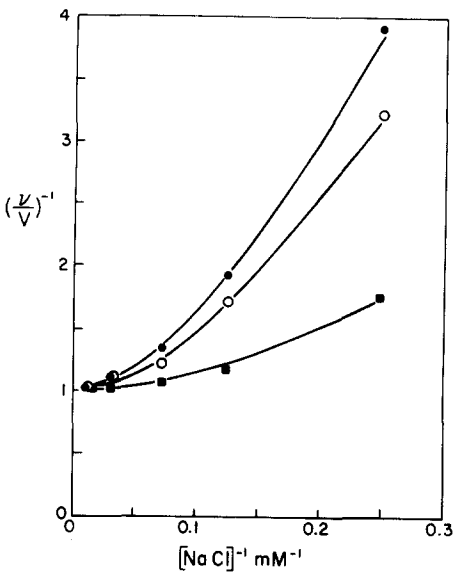


Fig. 3. Effects on Na^+-activation. Experiments were performed and data are presented as in Fig. 2, except that the KCl concentration was 10 mM whereas the NaCl concentrations were as shown: in the absence of inhibitors (●), or with oligomycin, 10 μg/ml (■), or quercetin, 2 μg/ml (○).

this enzyme the effects of the two reagents were also distinctly different: oligomycin did not inhibit in the standard assay medium whereas quercetin inhibited, if anything, more than with the (Na^+ + K^+)-ATPase activity (Tables I, II). ADP acts as a competitor toward the substrate of the K^+-phosphatase activity, nitrophenyl phosphate, also [18], and in the presence of 1 mM ADP oligomycin

TABLE II

INHIBITION OR STIMULATION OF K^+-PHOSPHATASE ACTIVITY

K^+-phosphatase activity was measured in the standard medium (see Methods), modified by the additions noted, in the absence and presence of oligomycin or quercetin. Percentage inhibition (−) or stimulation (+) is calculated relative to the corresponding control, treated identically except for the absence of inhibitor. In addition, activity with the various modifications is presented relative to that in the standard medium, all in the absence of oligomycin or quercetin.

Experimental conditions	Relative activity without oligomycin or quercetin	Percent change in activity	
		with oligomycin (10 μg/ml)	with quercetin (2μ/ml)
10 mM KCl	(100)	+2±1	−64±3
plus ADP, 1 mM	70	−13±1	−67±4
plus Me_2SO, 100 μl/ml	145	-	−6±2
0.3 mM KCl	14	−29±1	−62±4
plus Me_2SO, 100 μl/ml	28	−19±2	-
plus NaCl, 10 mM	10	+110±6	−68±3
plus NaCl, 10 mM, and CTP, 0.3 mM	78	−11±1	−96±1

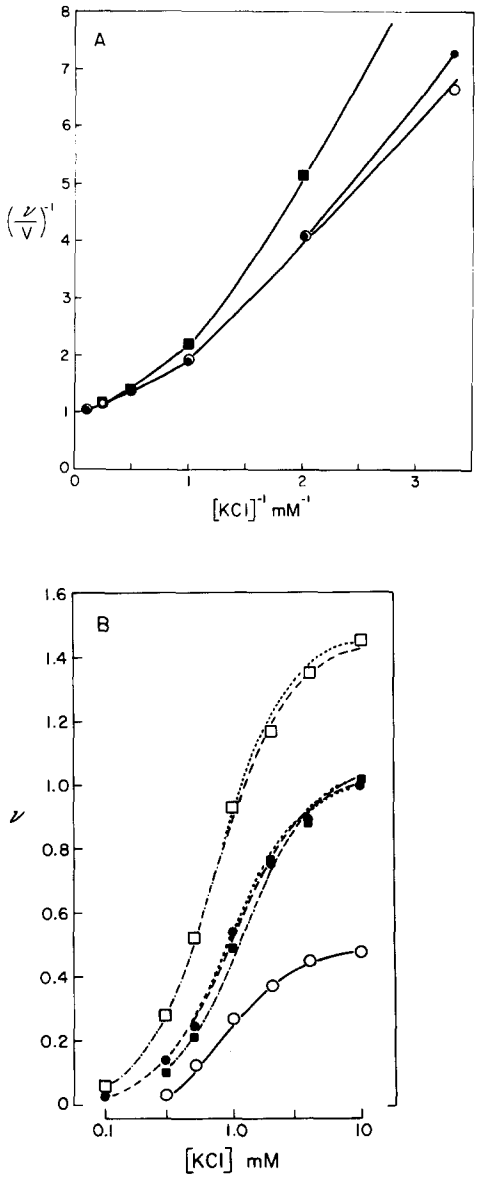


Fig. 4. K^+-activation of the phosphatase reaction. K^+-phosphatase activity was measured in the standard medium modified to contain the concentrations of KCl shown. In panel A velocities are plotted in double-reciprocal form relative to the maximal velocity, from experiments in the absence of inhibitors (●), or with 10 μg/ml oligomycin, (■) or 2 μg/ml quercetin (○). In panel B these data are re-plotted relative to the velocity in the standard medium in the absence of inhibitors, defined as 1.0; in addition, velocities from experiments to which 100 μl/ml Me_2SO was added are also plotted (□). The dashed and dotted lines are calculated values using the model from Fig. 5 and sets (i) and (ii) of the constants, respectively, from Table III.

then inhibited slightly, although quercetin inhibited essentially to the same extent (Table II).

When the KCl concentration of the K^+-phosphatase assay medium was reduced well below the $K_{0.5}$ value, to 0.3 mM, then oligomycin clearly inhibited (Table II), although quercetin inhibited, if anything, less than with 10 mM KCl. Thus oligomycin, in contrast to its decreasing the $K_{0.5}$ for KCl as activator of the $(Na^+ + K^+)$-ATPase (Fig. 2), increased the $K_{0.5}$ of the K^+-phosphatase activity slightly (Fig. 4A, B), from 0.9 mM to 1.1 mM.

A previous model for the K^+-phosphatase reaction [12] related catalytic activity quantitatively to the concentration of E_2K_n, in terms of specified equilibrium constants between E_1 and E_2 conformational states in the absence and presence of bound K^+, and of the dissociation constant for K^+. An extension of this model (Fig. 5) includes substrate binding, and thus relates catalytic activity to the concentration of the K^+- and substrate-bound E_2 state, $E_2S \cdot K_n$. With the values for the constants listed in Table III, this extended model generates the broken lines of Fig. 4B. Under control conditions (absence of inhibitors), one set of constants, (i), generates the dashed line; this set was based on a value for K_3 of 5.0, near those previously chosen [12] and applied to the catalytic process [20]. Some constants, however, differ from those of the previous model [12], which contained within them contributions from K_5 and K_6. The other set of constants, (ii), generates the dotted line; this set was based on a value for K_3 of 100, within the range calculated from fluorescence studies of enzyme conformational states [21], and leads to a nearly identical curve through major changes in only one other parameter, K_2 (Fig. 4B; Table III).

This extended model can also account for the inhibition by oligomycin seen only at low KCl concentrations (Fig. 4B), through, chiefly, decreases in K_1 and K_3 (Table III). Such changes thus represent oligomycin-induced shifts toward E_1 conformational states (Fig. 5).

Me_2SO stimulates the K^+-phosphatase activity and reduces the $K_{0.5}$ for KCl, opposite to it effects on the $(Na^+ + K^+)$-ATPase activity [22]. These alterations in $K_{0.5}$ for K^+ and V_{max} for the K^+-phosphatase reaction can also be accommodated

TABLE III

VALUES OF CONSTANTS FOR CALCULATED VELOCITIES

These two sets of constants were used in the equations of Figs. 5 and 6 to calculate the dashed lines, using set (i), or the dotted lines, using set (ii), of Figs. 4, 7, and 8 and the calculated velocities of Table IV; Values were chosen empirically.

Constant	Experimental conditions					
	Control		With oligomycin, 10 μg/ml		With Me_2SO, 100 μl/ml	
	(i)	(ii)	(i)	(ii)	(i)	(ii)
K_1	0.7	0.7	0.6	0.6	5.0	5.0
K_2 (mM)	1.25	20.0	1.25	20.0	1.25	20.0
K_3	5.0	100.0	0.8	25.0	50.0	500.0
K_4 (mM)	1.25	1.25	1.25	1.25	1.25	1.25
K_5 (mM)	2.22	3.33	1.11	3.0	1.0	1.43
K_6 (mM)	1.5	1.5	1.5	1.5	0.8	0.8
n	1.55	1.6	1.55	1.6	1.55	1.6
m	1.4	1.4	1.4	1.4	1.4	1.4
p	1.5	1.5	1.5	1.5	1.5	1.5
K_K (mM)	0.15	0.15	0.05	0.05	0.2	0.2
K_{Na} (mM)	15.0	15.0	5.0	5.0	5.0	5.0
B	0.103	0.116	0.275	0.300	2.0	1.8

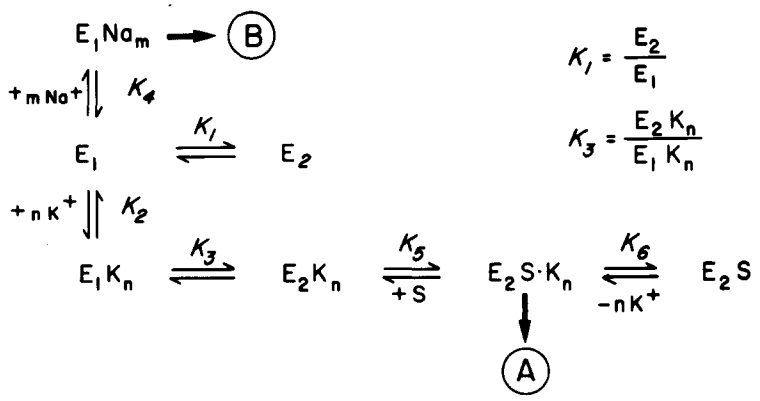
by the extended model (Fig. 4B), with either set of constants (Table III) through major increases in K_1 and K_3, representing shifts towards E_2 conformational states (Fig. 5). Moreover, Me_2SO almost eliminated inhibition by quercetin and reduced that by oligomycin (Table II), just as with the $(Na^+ + K^+)$-ATPase activity (Table I).

NaCl acts as a competitor to KCl at high concentrations [12], interpretable as a shift away from the active $E_2S \cdot K_n$ form of pathway A (Fig. 5). This inhibition, however, is reduced rather than increased at very low KCl concentrations, although even with 0.3 mM KCl activity without NaCl is greater then with it. Nevertheless, the inhibition is far less than that expected from effects with 10 mM KCl, and this discrepancy is interpretable as the appearance of an alternative $(Na^+ + K^+)$-stimulated pathway for the phosphatase reaction [11,12], represented in Fig. 5 as pathway B and related to the formation of E_1Na_m; even with Na^+, some K^+ is still required for activity [12]. Under such conditions, with 0.3 mM KCl and 10 mM NaCl, oligomycin greatly stimulated activity (Table II), as first demonstrated by Askari and Koyal [23]. By contrast, quercetin inhibited, if anything, even more (Table II).

Assuming as before [12] that the alternative pathway B is analogous to that of the $(Na^+ + K^+)$-ATPase reaction, with Na^+-activated phosphorylation of the enzyme and K^+-activated dephosphorylation (through high-affinity, extracellularly-oriented K^+-sites), then velocity over this pathway, v_B, would be proportional to $E_2 - P \cdot K_p$, as shown in Fig. 6. Total activity in the presence of K^+ and Na^+ would thus be the sum of activities over pathways A and B.

Measured total activities at various low concentrations of KCl and at NaCl concentrations from 0 to 30 mM, shown as the solid symbols in Fig. 7A, are fitted well by this model and either set of the constants of Table III, as shown by the calculated values connected by the broken lines. These calculated values represent the sum of v_A, using the constants previously evaluated (Table III), plus v_B, using the newly-evaluated apparent affinities for K^+ at its activating sites, K_K, and for Na^+ as a competitor at those sites, K_{Na} (Table III). The factor B relates the maximal velcoty over this pathway to the maximal velocity over pathway A; B thus includes the rates of Na^+-activated phosphorylation and of enzyme isomerization from $E_1 - P$ to $E_2 - P$.

The effects of oligomycin (Fig. 7B) and Me_2SO (Fig. 7C) on phosphatase activity in the presence of low concentrations of KCl and of 0–30 mM NaCl can also be accommodated by the model



$$E_2S\cdot K_n = \frac{K_3 K_4 [K^+]^n [S]}{K_2K_4K_5 + K_1K_2K_4K_5 + K_2K_5[Na]^m + [K^+]^n(K_4K_5 + K_3K_4K_5 + K_3K_4[S]) + K_3K_4K_6[S]}$$

Fig. 5. Model for enzyme conformations and K^+-phosphatase activity. A rapid equilibrium model for the pertinent conformations and ligand-bound states is shown, with enzyme velocity over pathway A, in the absence of Na^+, proportional to $E_2S\cdot K_n$, the E_2 conformation with bound substrate and K^+ (n being the Hill coefficient for cooperative interactions among K^+-sites). In the presence of Na^+ an alternative pathway, B, becomes available, as shown in further detail in Fig. 6.

(broken lines) with either set of constants (Table III). With oligomycin K_K is decreased, in accord with the decrease in $K_{0.5}$ for KCl in the ($Na^+ + K^+$)-ATPase reaction (Fig. 2), whereas with Me_2SO it is increased, in accord with the increase in $K_{0.5}$ in the ($Na^+ + K^+$)-ATPase reaction [20]; for both K_{Na} is decreased. B is increased nearly 3-fold with oligomycin, but almost 20-fold with Me_2SO.

In the absence of oligomycin and Me_2SO, phosphatase activity in the presence of 0.3 mM KCl and 10 mM NaCl was greatly increased by adding CTP (Table II), as previously shown [16]. Oligomycin inhibited somewhat, as previously described [10,11,23], whereas quercetin inhibited drastically (Table II).

Neither oligomycin nor quercetin affected the apparent K_m for the substrate, nitrophenyl phosphate, when measured with 10 mM KCl (Fig. 8). The model of Fig. 5 describes this relationship

$$E_1Na_m \overset{+S}{\rightleftharpoons} E_1S\cdot Na_m \rightleftharpoons E_1\text{-}P\cdot Na_m \rightleftharpoons E_2\text{-}P\cdot Na_m \overset{-mNa^+}{\rightleftharpoons} E_2\text{-}P \overset{+pK^+}{\rightleftharpoons} E_2\text{-}P\cdot K_p$$

$$v_B \propto [E_2\text{-}P\cdot K_p] = \frac{B[E_1S\cdot Na_m]}{1+\left\{\frac{K_k}{[K^+]}\left(1+\frac{[Na^+]}{K_{na}}\right)\right\}^p}$$

Fig. 6. Model for ($Na^+ + K^+$)-dependent phosphatase activity. A reaction sequence for pathway B is shown, with velocity proportional to $E_2 - P\cdot K_p$, the phosphorylated E_2 conformation with high-affinity extracellular K^+-sites at which Na^+ can compete (p being the Hill coefficient); the maximal velocity is related to that over pathway A by the factor B.

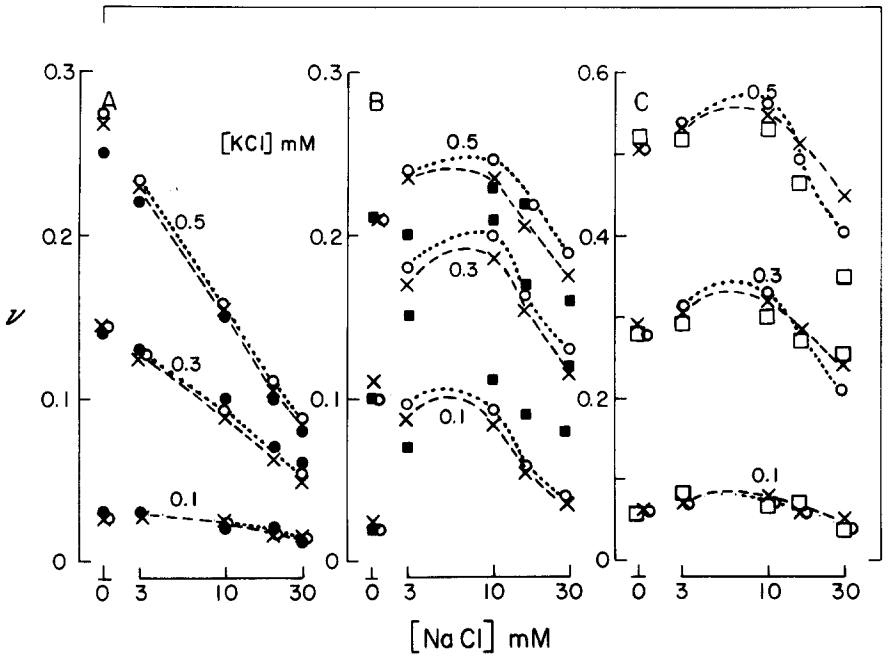


Fig. 7. Stimulation of $(Na^+ + K^+)$-dependent phosphatase activity by oligomycin and Me_2SO. Phosphatase activity was measured in the standard medium modified to contain either 0.5, 0.3, or 0.1 mM KCl, together with NaCl concentrations from 0 to 30 mM, as shown. In panel A are plotted control velocities (●); in panel B velocities from incubations with 10 μg/ml oligomycin (■); and in panel C velocities with 100 μl/ml Me_2SO (□). In all cases these velocities are plotted relative to that in the standard medium, with 10 mM KCl and no modifiers, defined as 1.0. The dashed and dotted lines, and symbols X and ○ represent calculated velocities for the total activity over both pathways A and B, using the model of Figs. 5 and 6 and the two sets of constants, respectively, of Table III.

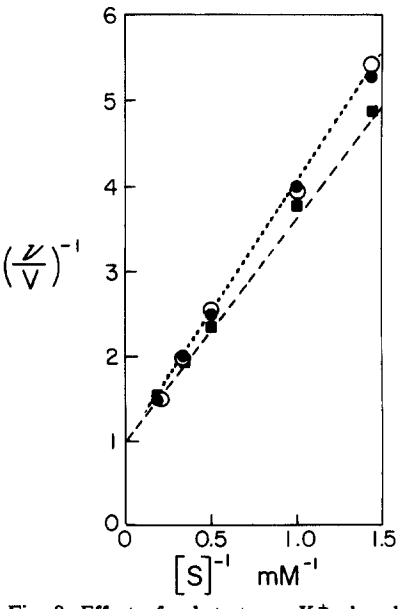


Fig. 8. Effect of substrate on K^+-phosphatase activity. K^+-Phosphatase activity was measured in the standard medium modified to contain the concentrations of nitrophenyl phosphate shown. Velocities are plotted in double-reciprocal form relative to the maximal velocity, from experiments in the absence of inhibitors (●), or with 10 μg/ml oligomycin (■), or 2 μg/ml quercetin (○). The dashed line is the calculated value for both the control and oligomycin experiments, using the model of Fig. 5 and set (i) of the constants of Table III. The dotted line is the calculated value for the control experiments, using set (ii) of the constants; for the oligomycin experiments the calculated line would fall between the dotted and dashed lines (Table IV).

well, as shown by the broken lines of Fig. 8 for both the absence and presence of oligomycin (using either set of the constants in Table III for control and for oligomycin conditions). When the KCl concentration was decreased, in the absence and presence of NaCl, the apparent K_m was affected, and these changes were modified by addition of oligomycin or Me_2SO. The observed ratio of velocity with 1 mM substrate to that with 3 mM, reflecting the apparent K_m, again can be fitted reasonably well by the model (Table IV), both for

TABLE IV

EFFECT OF SUBSTRATE CONCENTRATION ON OBSERVED AND CALCULATED K^+-PHOSPHATASE ACTIVITY

K^+-phosphatase activity was measured in the standard medium modified to contain either 3 mM or 1 mM nitrophenyl phosphate, the concentrations of monovalent cations shown, and the absence and presence of 10 μg/ml oligomycin or 100 μl/ml Me_2SO. The ratio of velocities with the two substrate concentrations is presented from these experiments, together with the calculated velocity over pathway A alone, or the sum of the velocities over both A and B; calculations are based on the model of Figs. 5 and 6 and the two sets of constants of Table III.

[KCl] (mM)	[NaCl] (mM)	Additions	Ratio: $\frac{\text{velocity with 1 mM substrate}}{\text{velocity with 3 mM substrate}}$				
			Observed	Calculated over pathway A		Calculated over Pathways A + B	
				(i)	(ii)	(i)	(ii)
10	0	none	0.50 ± .02	0.52	0.49	–	–
		oligomycin	0.52 ± .02	0.52	0.51	–	–
		Me_2SO	0.57 ± .03	0.67	0.61	–	–
0.5	0	none	0.64 ± .02	0.67	0.63	–	–
		oligomycin	0.61 ± .03	0.56	0.51	–	–
		Me_2SO	0.69 ± .02	0.80	0.72	–	–
0.5	10	none	0.58 ± .02	0.42	0.40	0.44	0.68
		oligomycin	0.52 ± .04	0.36	0.36	0.55	0.65
		Me_2SO	0.63 ± .03	0.69	0.58	0.70	0.71
0.3	0	none	0.73 ± .02	0.70	0.67	–	–
		oligomycin	0.64 ± .04	0.56	0.52	–	–
		Me_2SO	0.78 ± .03	0.84	0.75	–	–

control conditions and with oligomycin and Me_2SO, using either set of constants (Table III). These calculations also demonstrate again the necessity for invoking activity over pathway B in addition to the activity of pathway A when NaCl was present (Table IV).

Discussion

With a reaction sequence for the $(Na^+ + K^+)$-ATPase requiring cyclical interconversions between E_1 and E_2 conformations, it might seem that, if reagents like oligomycin and quercetin block the transitions similarly, then they should affect catalytic activity similarly. Correspondingly, it might seem that, with ligands binding predominantly to E_1 conformations (like ATP, ADP, and Na^+) or to E_2 conformations (like K^+ and nitrophenyl phosphate), the effects on ligand binding and of ligands on the reagent-induced changes should then be similar. Nevertheless, the disparate effects of oligomycin and quercetin described here may still be largely accommodated within a similar framework. A more detailed examination of the actions of oligomycin does, however, reveal the complexities both of such interactions and of the catalytic mechanisms as well.

For quercetin, presumed to favor E_1 conformations [13], the apparent insensitivity of inhibition to the concentrations of ligands that affect such transitions oppositely (e.g., K^+ and nitrophenyl phosphate: Figs. 2, 4 and 8) or similarly (e.g., ATP and Na^+: Figs. 1 and 3) may simply reflect the slowly-reversible nature of this inhibition. The response to these ligands would then follow the observed (apparently) non-competitive pattern rather than the competitive pattern expected with a reversible inhibitor. That Me_2SO strongly reduced inhibition by quercetin (as well as by oligomycin) may be explained by Me_2SO not only favoring E_2 conformations [12] but also promoting quercetin dissociation (unfortunately, in the absence of direct measurements of quercetin binding this possibility cannot be assessed). The lesser

inhibition by quercetin in the presence of ADP (Table I) could result from antagonism between the binding of these two inhibitors, but the greater inhibition of $(Na^+ + K^+)$-CTPase activity compared to $(Na^+ + K^+)$-ATPase is not readily explainable. The lower V_{max} with CTP is attributable to its poorer efficacy as a selector of E_1 conformations and thus its poorer facilitation of the E_2 to E_1 transition in which bound nucleotides participate [19]. Quercetin, by favoring E_1 transitions, should inhibit equally with ATP or CTP the $E_1 - P$ to $E_2 - P$ transition that presumably occurs in the absence of bound nucleotides; conversely, quercetin should facilitate the E_2 to E_1 transition, and this action, with the potential for lessening overall inhibition, should thus be more prominent with the less-effective nucleotide, CTP.

For oligomycin some responses are readily explained by its favoring E_1 conformations, including the lesser inhibition in the presence of Me_2SO and the lesser inhibition of the $(Na^+ + K^+)$-CTPase activity (Table I). The decrease in K_m for ATP and $K_{0.5}$ for Na^+ are consistent with such an action as well, although it is also possible that oligomycin binds preferentially to $E_1 - P$ producing an uncompetitive pattern of inhibition [24–26]. Analogously, the greater inhibition by oligomycin in the presence of ADP can be attributed to synergistic inhibition by non-exclusive inhibitors. The decrease in $K_{0.5}$ for K^+ (Fig. 2), on the other hand, cannot be so readily explained. Sachs [26] proposed an uncompetitive form of inhibition to account for the reduction in $K_{0.5}$ for K^+, although here the necessary intervening irreversible step is absent: although Na^+ may be released before K^+ binds, Na^+ is present at high concentrations and thus product release is not irreversible as it is in conventional studies of initial velocities. The complexities of the $(Na^+ + K^+)$-ATPase reaction hinder a detailed quantitative assessment of alternative proposals, but the processes may be illuminated by examining a more tractable enzymatic activity, the K^+-phosphatase reaction.

With the K^+-phosphatase reaction, which seems to be catalyzed by E_2 conformations [12], both oligomycin and quercetin should be potent inhibitors: the potential stimulation of the $(Na^+ + K^+)$-ATPase reaction through facilitating its E_2 to E_1 transition thus being irrelevant. Indeed, quercetin inhibited the K^+-phosphatase more than the $(Na^+ + K^+)$-ATPase reaction, this inhibition was diminished by Me_2SO, and the kinetic pattern was consistent with a slowly-reversible inhibitor. Oligomycin, on the other hand, did not inhibit K^+-phosphatase activity under standard assay conditions (Table II), although inhibition did occur at quite low K^+-concentrations (Fig. 4).

These effects of oligomycin can, however, be accommodated by an extension (Fig. 5) of a recent model for the K^+-phosphatase reaction [12], with oligomycin shifting equilibria toward E_1 conformational states (Table III). As before, this model makes the simplifying and plausible assumption for the K^+-phosphatase reaction of rapid equilibrium [12]. Unique evaluation of the interrelated constants is not possible, but the constraints of fitting the variety of experimental conditions lend credence to the general form of the model. The similar results with two widely different values chosen for K_3 demonstrate that changes in this parameter have major consequences only for the derived value of K_2.

Unfortunately, appropriate evaluations of none of the equilibrium constants, including K_3, are available. Karlish [27], from experiments with a fluorescent labeled-enzyme, calculated a value for the E_2K/E_1K equilibrium near 1000, whereas Beaugé and Glynn [21], measuring intrinsic protein fluorescence, found values ranging from 52 to 523; these studies measured equilibria at 19–20°C in the absence of divalent cations, and neither considered E_2/E_1 equilibria. Using a different label and a different model for the transitions, Esmann and Skou [28] calculated the equilibrium between E_2K(occluded) and E_2K to be near 300. On the other hand, Hobbs et al. [20] fitted catalysis at 21°C in the presence of divalent cation to a model with an E_2K/E_1K equilibrium of 8. Since E_1 states are favored at higher temperatures [29], the two values chosen here for K_3, 100 and 5, seem reasonable approximations to these diverse reports. Values for the E_2/E_1 equilibrium are even less readily obtained from previous studies. Early reports assumed the equilibrium lay far toward E_1, but later studies showed the dependence on buffer composition and pH: in the absence of histidine and at lower pH values the equilibrium lies toward E_2 [30]. Consequently, a choice for K_1 of 0.7

seems, under the present circumstances, reasonable.

Thus, using either set of constants (Table III), the observed effects of both oligomycin and Me_2SO on V_{max}, $K_{0.5}$ for K^+, and K_m for substrate are fitted quantitatively (Fig. 4; Table IV), with effects of oligomycin manifested through major shifts in equilibria toward E_1 conformational states. Attempts to fit these data with fewer than five equilibrium constants (K_1, K_2, K_3, K_5, and K_6) and one exponent (n) were unsuccessful; clearly, no more parameters were necessary to accommodate these data satisfactorily.

In the presence of Na^+, however, the responses of the phosphatase activity became more complex, requiring more parameters. The earlier proposal [11,12] of an emergent ($Na^+ + K^+$)-dependent pathway (Figs. 5 and 6: pathway B) providing an alternative catalytic sequence for nitrophenyl phosphate hydrolysis can, however, account for the observed velocity as the sum of activities through pathways A and B. The model (Figs. 5 and 6) describes reasonably well inhibition and stimulation by Na^+, oligomycin, and Me_2SO, and the effects on $K_{0.5}$ for K^+ and K_m for substrate in the presence of NaCl (Fig. 7; Table IV). These calculations use the same sets of constants as before, but are now augmented with K_4 and m as well as those of pathway B, which depict competition between Na^+ and K^+ for the high-affinity extracellularly-oriented K^+-sites of pathway B [12] plus the maximal velocity B over pathway B.

How Me_2SO can stimulate pathway B, since it favors E_2 conformations and increases the $K_{0.5}$ for K^+ at the high-affinity extracellularly-oriented sites, is accounted for by the 20-fold increase in B, and this may be plausibly attributed to its favoring the $E_1 - P$ to $E_2 - P$ transition necessary in that pathway [12]. How oligomycin can stimulate pathway B might seem obvious, since it favors E_1 conformations. Quantitative assessment of this effect, however, demonstrates that such a process cannot account for the stimulation by oligomycin. With 10 mM NaCl and 0.3 KCl the enzyme is already overwhelmingly in the E_1 conformations as calculated by any values for the constants consistent with the rest of the data. A 3-fold increase in B is thus required to fit the data, but by what mechanism this may result is uncertain, especially since oligomycin inhibits the necessary $E_1 - P$ to $E_2 - P$ transition. Indeed, inhibition of the Na^+ plus CTP-induced stimulation of the phosphatase reaction by oligomycin (Table II) can be explained simply by such inhibition of $E_2 - P$ formation and the failure to form the high-affinity extracellularly-oriented K^+-sites. Recently, Hobbs et al. [7] showed that oligomycin slowed down enzyme phosphorylation by ATP in the presence of K^+, indicating a diminished formation of $E_1 - P$ and making the postulated increase in B even less explicable if nitrophenyl phosphate interacts as does ATP. Thus, although the model can accommodate the data well, the interpretation of the required magnitudes for the constants indicates that if the model is basically valid some further elaborations or modifications are still required, such as facilitated Na^+-stimulated phosphorylation, or heterogeneity of cation sites, or subunit interactions within an oligomeric enzyme.

Acknowledgement

This work was supported by U.S. Public Health Service research grant NS-05430.

References

1 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–176
2 Cantley, L.C. (1981) Curr. Topics Bioenerg. 11, 201–237
3 Schuurmans Stekhoven, F. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76
4 Fahn, S., Koval, G.J. and Albers, R.W. (1966) J. Biol. Chem. 241, 1882–1889
5 Karlish, S.J.d., Yates, D.W. and Glynn, I.M. (1978) Biochim. Biophys. Acta 525, 252–264
6 Skou, J.C. (1982) Biochim. Biophys. Acta 688, 369–380
7 Hobbs, A.S., Albers, R.W. and Froehlich, J.P. (1983) J. Biol. Chem. 258, 8163–8168
8 Post, R.L., Toda, G. and Rogers, F.N. (1975) J. Biol. Chem. 250, 691–701
9 Israel, Y. and Titus, E. (1967) Biochim. Biophys. Acta 139, 450–459
10 Askari, A. and Koyal, D. (1968) Biochem. Biophys. Res. Commun. 32, 227–232
11 Robinson, J.D. (1970) Arch. Biochem. Biophys. 139, 164–171
12 Robinson, J.D., Levine, G.M. and Robinson, L.J. (1983) Biochim. Biophys. Acta 731, 406–414
13 Kuriki, Y. and Racker, E. (1976) Biochemistry 15, 4951–4961
14 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52

15 Robinson, J.D. (1967) Biochemistry 6, 3250–3258
16 Robinson, J.D. (1969) Biochemistry 8, 3348–3355
17 Hexum, T., Samson, F.E., Jr. and Himes, R.H. (1970) Biochim. Biophys. Acta 212, 322–331
18 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006–1019
19 Robinson, J.D. (1982) Arch. Biochem. Biophys. 213, 650–657
20 Hobbs, A.S., Albers, R.W. and Froehlich, J.P. (1980) J. Biol. Chem. 255, 3395–3402
21 Beaugé, L.A. and Glynn, I.M. (1980) J. Physiol. 299, 367–383
22 Robinson, J.D. (1972) Biochim. Biophys. Acta 274, 542–550
23 Askari, A. and Koyal, D. (1971) Biochim. Biophys. Acta 225, 20–25
24 Inturrisi, C.E. and Titus, E.O. (1968) Mol. Pharmacol. 4, 591–599
25 Robinson, J.D. (1971) Mol. Pharmacol. 7, 238–246
26 Sachs, J.R. (1980) J. Physiol. 302, 219–240
27 Karlish, S.J.D. (1980) J. Bioenerg. Biomembranes 12, 111–136
28 Esmann, M. and Skou, J.C. (1983) Biochim. Biophys. Acta 748, 413–417
29 Swann, A.C. (1983) Arch. Biochem. Biophys. 221, 148–157
30 Skou, J.C. and Esmann, M. (1980) Biochim. Biophys. Acta 601, 386–402