Synergistically Stimulated (Na⁺,K⁺)-Adenosine Triphosphatase from Plasma Membrane of a Marine Diatom

(*Nitzschia alba*)

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ABSTRACT An ATP-hydrolyzing activity with the properties of a Mg²⁺-dependent (Na⁺,K⁺)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from a 20-fold purified plasma membrane fraction of the marine diatom, *Nitzschia alba* is described.

The basal activity requires Mg²⁺ and further stimulation by Na⁺ or Na⁺ plus K⁺ is dependent on the presence of Mg²⁺; Mn²⁺ or Co²⁺ can partially substitute for the divalent cation requirement but Ca²⁺ equimolar with Mg²⁺ inhibits the activity by 54%. ATP is the preferred substrate for the Na⁺ plus K⁺ stimulated activity, while CTP, UTP, and ADP are only slightly hydrolyzed. The apparent K_m is 8 × 10⁻⁴ M ATP.

The ATP hydrolysis-rate is dependent on the relative concentrations of Na⁺ and K⁺; the K_{0.5} for Na⁺ and K⁺ are 2 mM and 50 mM, respectively. Basal activity is synergistically stimulated by Na⁺ plus K⁺ only at certain ion concentrations and shows a strong specificity for both cations.

In the presence of Na⁺ at 5 mM and K⁺ at 350 mM, the ATPase is completely inhibited by *p*-chloromercuric benzoic acid 10⁻⁴ M, *N*-ethyl maleimide 10⁻³ M, and iodoacetamide 10⁻³ M, but is insensitive to ouabain at 10⁻⁷ to 10⁻³ M.

This study demonstrates for the first time that algal plasma membrane contains an ATPase that is synergistically stimulated by Na⁺ and K⁺.

Definitive evidence for the presence in plant or algal membranes of a Mg²⁺-dependent adenosine triphosphatase [ATP phosphohydrolase (ATPase) EC 3.6.1.3] which is synergistically stimulated by Na⁺ plus K⁺ has not to our knowledge been presented. There have been several reports (1-5) of a (Na⁺,K⁺)-activated ATPase, derived from plant or algal cells; in most cases it is described as resembling the "transport" (Na⁺,K⁺)-ATPase of animal cells (6-8). The evidence, however, is not convincing.

In accordance with Skou's definition (6), we propose the following minimal criteria for a Na⁺ plus K⁺ synergistically stimulated ATPase which could be involved in active-linked transport of Na⁺ and K⁺ in plants or algae. The activity should: (1) be located in the cell membrane, (2) preferentially catalyze the hydrolysis of ATP as compared with other nucleotides, (3) show a Na⁺ plus K⁺ stimulation which is dependent on the presence of Mg²⁺, (4) show more stimulation with Na⁺ plus K⁺ than the total obtained with each ion separately, i.e., synergistic stimulation, (5) catalyze ATP-hydrolysis at a rate dependent on the relative concentrations

Abbreviations: PCMB, *p*-chloromercuric benzoic acid; NEM, *N*-ethyl maleimide; IAA, iodoacetamide; PCP, pentachlorophenol; ATPase, adenosine triphosphatase.

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of Na⁺ and K⁺, (6) have an affinity for Na⁺ different from that for K⁺, and (7) show specificity for these monovalent cations.

This paper reports the characteristics of such an ATP-hydrolyzing activity in the plasma membrane fraction of the marine diatom, *Nitzschia alba*.

METHODS

The Mg²⁺-dependent (Na⁺,K⁺)-ATPase preparation consisted of protoplast plasma-membranes, purified 20-fold, as described previously (9, 10).

ATPase activity was measured by the release of P_i and protein was determined by the method of Lowry, *et al.* (11) as described previously (9).

Nucleotides were purchased from Sigma Chemical Co. as the Tris salts. All chemicals were reagent grade and all solutions were prepared in deionized water that had been redistilled in an all-glass still.

RESULTS

The ion response of the ATP-hydrolyzing activity in the plasma membrane is highly influenced by Mg²⁺, Na⁺, and K⁺ (Table 1). In the absence of all three cations, there is almost no hydrolysis; what little there is presumably results from the small amount of alkaline phosphatase present in these membranes (9). Significant stimulation by Na⁺ and by Na⁺

TABLE 1. Magnesium dependence of the sodium and potassium ion stimulation of ATPase activity from plasma membranes of *Nitzschia alba*

Divalent cation (mM)	Na ⁺ /K ⁺ (mM)	Total ATPase activity (specific activity)	Net Na ⁺ ,K ⁺ -stimulated ATPase activity (specific activity)
None	0/0	0.53	—
	50/0	1.16	0.63
	0/50	0.72	0.19
	25/25	1.16	0.63
	5/45	0.47	-0.06
MgCl ₂	0/0	2.19	—
	50/0	10.3	8.11
	0/50	3.03	0.84
	25/25	10.3	8.11
	5/45	10.7	8.51

Specific activity, μmol P_i/mg of protein per hr.

TABLE 2. The effects of divalent cations on the Mg^{2+} -dependent (Na^+ , K^+)-ATPase from plasma membranes of *Nitzschia alba*

Divalent cations (2 mM)	Total ATPase activity (specific activity)	Net Na^+ , K^+ -stimulated ATPase activity (specific activity)
Mg^{2+}	6.16	3.97
Mn^{2+}	4.66	2.16
Ca^{2+}	2.84	0
Co^{2+}	1.97	1.26
Zn^{2+}	1.16	0
Cu^{2+}	0.06	0

plus K^+ occurs only in the presence of Mg^{2+} ions, while K^+ , either alone or in the presence of Mg^{2+} , has no significant effect.

The (Na^+ , K^+)-ATPase is preferentially stimulated by Mg^{2+} , but Mn^{2+} , and to a lesser extent Co^{2+} , can partially substitute for Mg^{2+} (Table 2).

With Mg^{2+} as the divalent cation, the (Na^+ , K^+)-stimulated activity showed a strong preference for ATP over other nucleotides tested (Table 3). Hence this activity is a "true" ATPase as has previously been suggested (10, 12).

The effect of a variety of compounds on the (Na^+ , K^+)-ATPase activity is shown in Table 4. Sulfhydryl group blocking agents, *p*-chloromercuric benzoic acid (PCMB), *N*-ethyl maleimide (NEM), and iodoacetamide (IAA), completely inhibited the Na^+ , K^+ -stimulated portion of the ATPase activity at high concentration (10^{-4} , 10^{-3} , and 10^{-2} M, respectively). The activity was especially sensitive to PCMB which gave 53% inhibition at a concentration of only 1×10^{-6} M, indicating the importance of the sulfhydryl group to the function of this enzyme. Pentachlorophenol (PCP), oligomycin, and antimycin A were strongly to moderately inhibitory whereas NaN_3 and ouabain were without effect. Calcium ions added at equimolar concentrations with magnesium ions produced a 54% inhibition.

Various aspects of the synergistic relationship of Na^+ / K^+ are clearly demonstrated by the data presented in Figs. 1-4. In the presence of low Na^+ (5 mM) half-maximal stimulation of the relative ATPase activity occurs at 50 mM K^+ while greatest stimulation, 7.5-fold, is reached at 200 mM K^+ ; above that, increased K^+ concentrations result in a sharp drop in activity (Fig. 1).

TABLE 3. Nucleotide substrate specificity of Mg^{2+} -dependent (Na^+ , K^+)-ATPase from plasma membranes of *Nitzschia alba*

Nucleotides (4 mM)	Total ATPase activity (specific activity)	Net Na^+ , K^+ -stimulated ATPase activity (specific activity)
ATP	6.90	5.50
ADP	0.43	0.38
AMP	0.09	0.09
CTP	1.33	0.87
ITP	1.48	-0.21
GTP	0.19	-0.48
UTP	0.90	0.38

TABLE 4. Effects of inhibitors on Mg^{2+} -dependent (Na^+ , K^+)-ATPase activity from plasma membranes of *Nitzschia alba*

Compound*	Concentration	Inhibition of Na^+ , K^+ - stimulated ATPase activity %
None	—	0
PCMB	1×10^{-4} M	100
	1×10^{-6} M	53
NEM	1×10^{-3} M	100
	1×10^{-6} M	53
IAA	1×10^{-2} M	100
NaN_3	1×10^{-3} M	0
PCP	4×10^{-4} M	88
Oligomycin	10 μ g/ml	25
Antimycin A	1×10^{-5} M	17
Ouabain	10^{-7} to 10^{-3} M	0
$CaCl_2$	2×10^{-2} M	54

* Abbreviations: PCMB, *p*-chloromercuric benzoic acid; NEM, *N*-ethyl maleimide; IAA, iodoacetamide; PCP, pentachlorophenol

At constant K^+ concentrations varied concentrations of Na^+ produce a very different response (Fig. 2). In the absence of K^+ , maximal stimulation of activity reaches as much as 15-fold at 200 mM Na^+ , after which activity steadily declines with increased Na^+ concentrations. In the absence of Na^+ , on the other hand, increasing K^+ concentra-

FIG. 1. Effects on ATPase activities of varied K^+ concentration at constant 5 mM Na^+ . Data are the average of three separate experiments. The standard assay medium contained 100 mM Tris-HCl (pH 8.5), 2 mM $MgCl_2$, 4 mM ATP (as the Tris salt; stock solution adjusted to pH 6.8 with 1 M Trizma base), NaCl, and KCl, as specified in the text and legends, and the enzyme preparation (10-50 μ g of protein per 1.0 ml of assay mix). Incubation was for 15-30 min at 30°; activity was linear with time for the duration of the assay. One unit of enzyme activity refers to 1 μ mol of P_i liberated per hour under the above assay conditions. The activity in the presence of Mg^{2+} alone, i.e., "basal (Mg^{2+})-ATPase," was measured concurrently. In the five plasma membrane preparations used for this study, the basal activity varied from 1.7 to 2.2 units/hr. A relative unit is equivalent to the specific activity of the ATPase assayed in the presence of Mg^{2+} only; thus, the increase in activity resulting from addition of Na^+ and K^+ is normalized for each experiment at the basal (Mg^{2+})-ATPase activity.

FIG. 2. Effects on ATPase activity of varied high Na^+ concentrations in the presence of constant K^+ ($\square$, $\circ$, $\bullet$), and of varied K^+ in the absence of Na^+ (Δ).

tions has almost no effect. However, with constant K^+ concentrations of 100 and 400 mM, and varied Na^+ concentrations two effects are noted. At the higher K^+ concentration, lower concentrations of Na^+ produce a more rapid rate of ATP hydrolysis; however, at that K^+ concentration the maximal activity attained is less than that produced by Na^+ alone or by Na^+ and 100 mM K^+ . Increased Na^+ concentrations above that required for maximal ATPase activity result in a decline in activity. Fig. 3 shows in more detail that the higher the amount of K^+ , the more rapidly does the ATPase activity (relative units) increase with increased Na^+ . Based on a $V_{\max}$ of 15 units/hr (Fig. 2), the half-maximal concentrations ($K_{0.5}$) for Na^+ decrease with increasing K^+ concentrations to 2 mM Na^+ at 350 mM K^+ and then begin to increase at higher K^+ concentrations (Fig. 3, insert). This indicates

FIG. 3. Effects on the ATPase activity of varied low Na^+ concentrations in the presence of constant K^+ concentrations. The inset depicts the half maximal Na^+ concentration ($K_{0.5}$) for stimulation of ATPase activity at varied K^+ levels; data calculated from Figs. 2 and 3.

FIG. 4. Synergistic stimulation of ATPase activity by various molar ratios of Na^+ and K^+ at constant ionic strength. The data represent average values from five separate experiments.

that the affinities of this ATPase system for Na^+ and K^+ differ and, furthermore, that the affinity for Na^+ changes as a function of the K^+ concentration.

A second major point demonstrated in Figs. 2 and 3 is that at Na^+ concentrations less than 50 mM and at K^+ concentrations up to 350 mM, the rate of ATP hydrolysis is greater when the ions are present together than when either is present singly, indicating that the combined effects of Na^+ plus K^+ are synergistic.

Synergistic stimulation is further illustrated in Fig. 4 where the stimulation by various Na^+/K^+ molar ratios, at constant ionic strength, is compared with that of each ion alone. At concentrations of 0–50 mM, K^+ alone has almost no effect, while with Na^+ alone, activity increases linearly with rising concentrations. However, when Na^+ plus K^+ are added to the assay mixture, the increase in relative stimulation is hyperbolic. Most importantly, these data clearly demonstrate that the stimulation resulting when Na^+ and K^+ are combined is greater than the sum of the stimulation produced by each separately. It is important to point out that the synergistic effect is greater at low concentrations of Na^+ . This further verified the observation that the stimulation of the ATPase activity is not the result of ion strength conditions prevailing in the assay medium. This defines the synergistic stimulation of the Mg^{2+} -dependent (Na^+/K^+)-ATPase by Na^+ plus K^+ . Figs. 2 and 4 indicate that as Na^+ concentrations are increased from 5 to 50 mM the stimulation resulting from K^+ addition diminishes.

To determine the monovalent cation specificity of the ATPase, various cations were tested at 45 and 350 mM in the presence of 5 mM Na^+ and at 5 mM in the presence of 350 mM K^+ (Table 5). The preferred cation, at 45 and 350 mM, is K^+ . In the presence of 350 mM K^+ , Na^+ is highly preferred. The more stringent requirement for Na^+ as compared with K^+ demonstrated here, is typical of the monovalent cation requirement of animal cell ATPase (3, 13). What is more important, however, is that the activating effects of Na^+ and K^+ are specific for these monovalent cations.

The effect of ATP concentration on ATPase specific activity under different ionic conditions is shown in Fig. 5; activity in the presence of 2 mM Mg^{2+} is stimulated more than 5-fold by 5 mM Na^+ plus 45 mM K^+ and almost 10-fold by 5 mM Na^+ plus 350 mM K^+ . To determine whether this increase in specific activity with increased concentrations of K^+ was entirely the result of the increased affinity for Na^+ ($K_{0.5}$) (Fig. 3, insert), or whether an altered K_m for the substrate ATP might also be responsible, Lineweaver-Burk plots were

TABLE 5. Monovalent cation specificity of Mg^{2+} -dependent (Na^+, K^+) -ATPase from plasma membranes of *Nitzschia alba*

Mono-valent cation	5 mM Na^+ + 45 mM cation		5 mM Na^+ + 350 mM cation		5 mM cation + 350 mM K^+	
	Specific activity	Control %	Specific activity	Control %	Specific activity	Control %
None	3.16*	100	3.17*	100	3.16†	100
Li^+	3.83	122	3.74	118	3.28	104
Na^+	—	—	—	—	18.2	576
K^+	7.10	225	18.1	571	—	—
Rb^+	5.19	164	3.83	120	3.82	120
Cs^+	4.44	140	1.09	34	4.55	144
NH_4^+	5.75	182	10.88	343	4.55	144
Choline	4.20	132	11.49	362	3.46	109

* Na^+ is present at 5 mM.† K^+ is present at 350 mM.

made of the activity versus substrate concentration (Fig. 6). The predominant K_m 's for the 5 mM Na^+ /45 mM K^+ and the 5 mM Na^+ /350 mM K^+ are 8.7×10^{-4} M and 8.0×10^{-4} M ATP, respectively (Fig. 6b and c) indicating that the K_m for ATP does not change significantly with the relative concentrations of Na^+ and K^+ . It is only the V_{max} which increases with increasing K^+ concentration, probably as a result of the decrease in $K_{0.5}$ for Na^+ as mentioned earlier.

The biphasic effect for the (Mg^{2+}) -ATPase (Fig. 6a) confirms our previous suggestion (10) that more than one basal Mg^{2+} -dependent ATPase is present in the plasma membrane fraction. One of these activities has a K_m of 1.0×10^{-3} M ATP, similar both to the K_m for the Mg^{2+} -dependent (Na^+, K^+) -ATPase (Fig. 6b) and to the higher K_m of the activity assayed with 5 mM Na^+ and 350 mM K^+ (Fig. 6c), while the second (Mg^{2+}) -ATPase activity was somewhat lower, $K_m = 1.7 \times 10^{-4}$ M ATP.

DISCUSSION

The existence of Mg^{2+} -dependent (Na^+, K^+) -ATPase in animal cell membranes has been extensively documented (6–8); however no report of a similar enzyme in plant or algal material has been sufficiently substantiated.

Hansson and Kylin (1) described an ATP hydrolyzing activity in the pellet fraction of sugar beet homogenate which

FIG. 5. Specific activity of ATPase at different ionic conditions of Na^+ and K^+ plotted against ATP concentration. FIG. 6. Lineweaver-Burk plots and kinetic constants of ATPase from data in Fig. 5. ATPase assayed in the presence of: (a) 2 mM Mg^{2+} only; (b) 2 mM Mg^{2+} , 5 mM Na^+ , 45 mM K^+ ; and (c) 2 mM Mg^{2+} , 5 mM Na^+ , 350 mM K^+ .

was stimulated by Mg^{2+} and further by a mixture of Na^+ plus K^+ or by K^+ alone. Since the results were not compared with those of the ions added singly, a truly synergistic stimulation of Na^+ plus K^+ was not demonstrated. A similar activity was also reported by Kylin and Gee (3) for leaves of the mangrove.

Lai and Thompson (4) found a (Na^+, K^+) -stimulated ATP hydrolyzing activity in a plant membrane fraction. However, stimulation was only 4–33% greater than that produced by Na^+ alone; the monovalent cation stimulation did not require Mg^{2+} ; and the nucleotide specificity of the activity was not, apparently, determined. Other plant material has yielded presumptive monovalent cation activated-ATPase activities, which, on more careful analysis, were actually found to be salt-stimulated acid phosphatase activities (12).

In a very detailed study of the membranes of oat roots by Hodges and coworkers (14–16), several ATP hydrolyzing activities were identified. The plasma membrane ATPase activity exhibited a greater specificity for cations than for anions; however, the preparation did not show a Na^+ plus K^+ synergistic stimulation of the ATPase activity, while K^+ alone was the most effective cation for stimulation.

Our investigation demonstrates conclusively a Mg^{2+} -dependent (Na^+, K^+) -ATPase in algal plasma membrane, the activity of which is highly dependent on the relative concentrations of Mg^{2+} , Na^+ , and K^+ . The enzyme requires Mg^{2+} for basal activity, and while Na^+ linearly increases activity to 15-fold at 200 mM this occurs only in the presence of Mg^{2+} . Activity is significantly stimulated by K^+ only when both Mg^{2+} and Na^+ are present. The preference of this

ATPase for ATP as a substrate is in conformity with the nucleotide hydrolyzing capabilities of the similar enzyme in animal sources (6, 8) and with Hoffman's finding that intact human reticulocytes require ATP as substrate for Na^+ transport (17).

ATP-hydrolysis is 5 to 15 times greater when Na^+ and K^+ are present together at optimal molar ratios and concentrations than when either is present alone at these concentrations; stimulation of the basal (Mg^{2+})-ATPase was maximal at 5 mM Na^+ and 350 mM K^+ . To establish a synergistic stimulation by Na^+ and K^+ such data as the above are essential, and in their absence, the previous reports of synergistic stimulation in plant and algal preparations are unsubstantiated. Arbitrary choice, in such studies, of a single concentration of Na^+ and K^+ presents an obvious pitfall, as Figs. 3 and 4 demonstrate. Thus, Na^+ at high concentrations (200 mM) is capable of exerting a stimulation equivalent to Na^+ plus K^+ at optimum ratio and concentration. It is the relative molar ratios and final concentrations of both ions that are critical to the synergistic stimulation, in the same enzyme preparations (Figs. 3 and 4) and among enzymes from different sources (6).

One important difference between the (Na^+ , K^+)-ATPase systems of animal and diatom cells is that diatom cells have a higher affinity for Na^+ than for K^+ at ionic conditions of optimal stimulation whereas in animal cells the relative affinities are reversed. In the context of Skou's model (6), it appears that in our studies we are seeing the "inside sites" expressed, i.e., the sites normally exposed to the high K^+ and low Na^+ concentrations expected for cytoplasm. We have not investigated the "inside-outside" orientation of the vesicles used in the present study.

The $K_{0.5}$ for K^+ of 50 mM (with Na^+ at 5 mM) is much higher than that reported for the (Na^+ , K^+)-ATPase of animal cells (6, 8), and suggests that the algal enzyme system has a much lower K^+ affinity. However, the comparatively high half-maximal concentrations of K^+ and Na^+ of the diatom enzyme are not surprising, since a marine algal cell is normally bathed in sea water (about 470 mM Na^+ and 10 mM K^+) and might have cytoplasmic concentrations of Na^+ and K^+ of 125 mM and 461 mM, respectively, as was shown for the diatom *Coscinodiscus walsbyi* (18). These lower affinities might be expected in an enzyme system sensitive to changes at high ionic concentrations such as would be involved in active linked transport of Na^+ and K^+ across the cell membrane.

A second difference between the animal and diatom ATPase system lies in the linear response of the diatom enzyme to Na^+ concentrations up to 200 mM. In the classic study (13) of the (Na^+ , K^+)-ATPase from the leg nerve of the shore crab, low concentrations of Na^+ , in the absence of K^+ , increased enzyme activity several-fold; in other animals, however, addition of Na^+ in the absence of K^+ had no effect (6, 8).

The two ATPase systems differ in still another respect: the cardiac glycoside, ouabain, to which animal ATPase is highly sensitive (19, 20), has no inhibitory effect on enzyme activity in the diatom at concentrations of 10^{-7} to 10^{-3} M. Although much importance is attributed to the ouabain sensitivity of the animal ATPase, this need not apply to plant or algal systems, since ouabain has not been shown to specifi-

cally inhibit both ion transport and ion activated ATPase activity in plants or algae (16, 20). In fact, with one exception (16), no strong correlation between both the ion transport and ion-activated ATPase systems has been clearly established outside the animal kingdom. It should be noted however that strong circumstantial evidence for a linked K^+ influx/ Na^+ efflux system in the fresh water charophyte *Nitella translucens* has been reported (21) and that the K^+ component is inhibited 58% by 5×10^{-5} M ouabain.

Similarities between the diatom and animal ATPase systems are worth noting: both are inhibited by the SH blocking agents, PCMB and NEM, as well as by IAA (6), and both are greatly inhibited by oligomycin (22) and Ca^{2+} (13). In addition, both systems have in common a more strict requirement for Na^+ than for K^+ (6).

In sum, the data clearly demonstrate the presence of an ATPase system in algae which is similar in several important respects to the Mg^{2+} -dependent (Na^+ , K^+)-ATPase in animal cell membranes; since it conforms to the six criteria set out in the introduction, the synergistically stimulated activity of the diatom ATPase has the potential for being involved in the active linked transport of Na^+ and K^+ .

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