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Characterization of Nigericin-Stimulated ATPase from Sealed Microsomal Vesicles of Tobacco Callus[1]

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HEVEN SZE[2]
Departments of Botany and Biochemistry, University of Kansas, Lawrence, Kansas 66045

ABSTRACT

To understand the function and membrane origin of ionophore-stimulated ATPases, the activity of nigericin-stimulated ATPase was characterized from a low-density microsomal fraction containing sealed vesicles of autonomous tobacco (*Nicotiana tabacum* Linnaeous cv. Wisconsin no. 38) callus. The properties of KCl-stimulated, Mg-requiring ATPases (KCl-Mg,ATPase) were similar in the absence or presence of nigericin. Nigericin (or gramicidin) stimulation of a KCl-Mg,ATPase activity was optimum at pH 6.5 to 7.0. The enzyme was inhibited completely by *N,N'*-dicyclohexylcarbodiimide (10 μM), tributyltin (5 μM), and partially by vanadate (200 μM), but it was insensitive to fusicoccin and mitochondrial ATPase inhibitors, such as azide (1 mM) and oligomycin (5 μg/ml). The ATPase was more sensitive to anions than cations. Cations stimulated ATPase activity with a selectivity sequence of $NH_4^+ > K^+$, Rb^+, Cs^+, Na^+, $Li^+ > Tris^+$. Anions stimulated Mg, ATPase activity with a decreasing sequence of Cl^- = acetate > SO_4^{2-} > benzene sulfonate > NO_3^-. The anion stimulation was caused partly by dissipation of the electrical potential (interior positive) by permeant anions and partly by a specific ionic effect. Plant membranes had at least two classes of nigericin-stimulated ATPases: one sensitive and one insensitive to vanadate. Many of the properties of the nigericin-sensitive, salt-stimulated Mg,ATPase were similar to a vanadate-sensitive plasma membrane ATPase of plant tissues, yet other properties (anion stimulation and vanadate insensitivity) resembled those of a tonoplast ATPase. These results support the idea that nigericin-stimulated ATPases are mainly electrogenic H^+ pumps originated in part from the plasma membrane and in part from other nonmitochondrial membranes, such as the tonoplast.

Although, membrane-associated ATPases dependent on Mg^{2+} and sensitive to salts are ubiquitously present in higher plant tissues (3, 8–10, 12–15, 22, 25, 29, 30), the functional significance and membrane identity of microsomal ATPases are unclear. The ATP-hydrolyzing enzymes are interesting because of their possible role in active H^+ or ion transport (9, 20, 23). Recent *in vitro* studies support this idea (6, 7, 21, 26, 28). However, only a few of the ATPases can be identified by their specific properties.

Mitochondrial ATPase is identified by optimal activity at pH 9 and inhibition by oligomycin (9, 13) and azide (17). This ATPase depends on Mg for activity, is involved in oxidative phosphorylation, and pumps protons.

Of at least three ATPases associated with the microsomal membranes (13), only a plasma membrane ATPase (density 1.14–1.17 g/cc) is well characterized. The K^+-stimulated, Mg^{2+}-requiring ATPase (Mg^{2+},K^+-ATPase) is sensitive to DCCD,[3] DES (1), and vanadate, but insensitive to oligomycin (9, 14, 22). The enzyme has a pH optimum of 6.5 and is specifically stimulated by alkali-cations (29) but not anions (14, 22). However, NO_3^- inhibited the plasma membrane ATPase from oat roots (14) but not corn leaves (22). The K^+-stimulated ATPase has a K_m value for Mg-ATP of 0.38 (14) or 0.28 mM (22) in oat or corn leaves, respectively.

The type and identity of microsomal ATPases associated with membranes of densities 1.09 and 1.13 g/cc are not well understood. Plasma membranes from oat roots or pea epicotyl are also found at a density near 1.13 g/cc (3, 8). This fraction showed two ATPase activities: a pH 8.5 ATPase sensitive to anions (3, 8) and a pH 6.5 ATPase sensitive to cations (3). Isolated vacuolar membranes also have a salt-stimulated Mg,ATPase (*e.g.* 12, 15, 30, and references therein), which appear similar to the plasma membrane ATPase. A potential characteristic to distinguish membranes of tonoplast from plasma membrane or mitochondria is the insensitivity of tonoplast ATPase to vanadate and oligomycin or azide (30). However, it is not clear whether microsomal membranes include tonoplast as well as other nonmitochondrial membranes.

A Mg^{2+}-requiring, KCl-stimulated ATPase activity that is specifically stimulated by H^+ and K^+ ionophores was recently found in a low-density microsomal fraction of tobacco callus (27) and oat roots (4) similar to the findings made by Rungie and Wiskich (25). The results suggest that the enzyme is involved in H^+ or K^+ transport or both and that the ATPase is regulated by a chemical or electrical potential or both. This conclusion is supported by the finding that the enzyme (referred to as a nigericin-stimulated ATPase) is an electrogenic pump, probably transporting H^+ (28).

To understand the function and to determine the membrane identity of nigericin-stimulated ATPase, I have conducted a more detailed characterization of the ionophore-stimulated ATPase from autonomous tobacco tissue callus. In this paper, I have initially treated ATP hydrolysis stimulated by nigericin as an activity catalyzed by one type of enzyme. However, the possibility of several classes of ionophore-stimulated ATPases is considered since the low-density microsomal fraction contains several membrane types. Inasmuch as preparations of one membrane type are difficult to obtain, the properties of enzyme activities might serve as a sensitive indicator of the types of ionophore-stimulated ATPases. The results show that the same type(s) of enzyme catalyzed ATP hydrolysis in the absence or presence of nigericin. Many of the properties of the nigericin-stimulated ATPase of tobacco callus are similar to those of the plasma membrane

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[2] Present address: Department of Botany, University of Maryland, College Park, MD 20742.

[3] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbesterol; DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NPP, *p*-phosphate.

Table I. *ATPase Activities at Various Conditions*

Condition	ATPase Activity		
	Total	KCl-stimulated	Nigericin-stimulated
A. No salt	Unspecific ATPase		
B. KCl	Unspecific KCl-ATPase	B − A = ΔA unspecific KCl-ATPase	
C. $MgSO_4$	Mg-ATPase		
D. $MgSO_4$ + KCl	Mg,KCl-ATPase	D − C = ΔB KCl-Mg,ATPase	
E. $MgSO_4$ + nigericin	Mg-ATPase with nigericin		
F. $MgSO_4$ + KCl + nigericin	Mg,KCl-ATPase with nigericin	F − E = ΔC KCl-Mg,ATPase with nigericin	ΔC − ΔB = ΔD nigericin-stimulated ATPase

Mg^{2+},K^+-ATPase from other plant tissues. However, partial inhibition of ATPase activity by vanadate suggest that plant membranes have at least two classes of ion-pumping ATPases.

MATERIALS AND METHODS

Materials. Autonomous tobacco callus tissue obtained from the stem pith of *Nicotiana tabacum* Linnaeous cv. Wisconsin no. 38 was used after 1 to 1.5 months growth on a basal medium without cytokinins or auxins.

Dextran T 70 (average mol wt 70,000) was obtained from Pharmacia Fine Chemicals. Oligomycin, DES, DCCD, EDAC, CMC, and DIDS were purchased from Sigma Chemical Co. Sodium vanadate (ortho) was obtained from Fisher. Nigericin was a gift from J. Berger and J. W. Westley of Hoffmann-La Roche. Fusicoccin was a generous gift from M. Ragazzini and G. Michieli of Montedison S. p. A. in Milan, Italy. K-benzene sulfonate was a gift from D. L. Hendrix. All other chemicals were reagent grade.

Preparation of Sealed, Low-Density Microsomal Vesicles. Sealed microsomal vesicles from tobacco callus were prepared by flotation of a crude microsomal fraction over a dextran cushion similar to the method described by Sze (27). Callus tissue was homogenized in 25 mM Tris-Hepes at pH 7.3, 250 mM sucrose, 3 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 1 mM DTT. The mixture was filtered and the homogenate was centrifuged for 10 min at 500*g*. The supernatant was centrifuged at 8,000*g* for 10 min to obtain the mitochondrial pellet. The pellet obtained by centrifuging the resulting supernatant at 13,000*g* for 15 min was discarded. The 13,000*g* supernatant was centrifuged at 60,000*g* for 30 min to obtain the crude microsomal pellet. This fraction was resuspended in 250 mM sucrose/2.5 mM Tris-Hepes at pH 7.2, layered over a 10% dextran/250 mM sucrose/2.5 mM Tris-Hepes cushion, and centrifuged at 70,000*g* for 2 h. The interface collecting above the dextran cushion is the sealed, low-density microsomal vesicles (previously referred to as purified microsomal vesicles). These vesicles are relatively free of mitochondria as shown by the low activities of Cyt *c* oxidase and malate dehydrogenase (27).

ATPase Assay. ATPase activity was determined by measuring the release of Pi (10, 27) and the method is briefly described in the relevant legends. Since the hydrolytic activity at 37°C was linear with time up to 1 h, assays were done at 37°C for 30 to 45 min with 10 to 30 μg of protein/0.5 ml of reaction mixture. Standard reaction mixture contained 30 mM Tris-Hepes buffer and 3 mM ATP-Tris or substrate. The concentrations of $MgSO_4$, KCl, and nigericin (Nig) when present were 3 mM, 50 mM, and 2 μM, respectively. ATP (or substrate) hydrolysis was measured under various conditions and the ATPase activities were given the terms shown in Table I. KCl-stimulated, Mg-dependent ATPase will be abbreviated as KCl-Mg,ATPase. In general, the term ATPase will refer to the Mg-ATPase(s) sensitive to salt and nigericin. The relative amount of sealed vesicles and the specific ATPase activity varied from one preparation to another. Therefore, each experiment was conducted on the same day with the appropriate controls. Protein concentration was measured by the Lowry (18) method using BSA as the standard.

Table II. *Substrate Specificity of the Nigericin-Sensitive KCl-Phosphatase of Sealed Microsomal Vesicles from Tobacco Callus*

Phosphatase activity stimulated by 50 mM KCl was determined with 30 mM Hepes-Tris at pH 6.5 and 3 mM substrate in the absence or presence of 3 mM $MgSO_4$. Nigericin was 5 μM. Results are the average of two to five experiments.

Substrate	KCl-Phosphatase Activity		
	−Nigericin	+Nigericin	ΔNigericin
	μmol Pi/mg · h (%)		
ATP-Mg	3.9 (100)	7.0 (100)	3.1 (100)
GTP-Mg	2.4 (61)	2.7 (38)	0.3 (10)
CTP-Mg	1.3 (33)	1.5 (21)	0.2 (6)
UTP-Mg	1.7 (43)	1.4 (20)	−0.3 (0)
ADP-Mg	1.0 (26)	1.3 (18)	0.3 (10)
AMP-Mg	0 (0)	0 (0)	0 (0)
NPP-Mg	1.4 (36)	1.6 (23)	0.2 (6)
ATP	1.7 (43)	2.0 (28)	0.3 (10)
GTP	1.2 (31)	ND[a]	
ADP	1.3 (33)	ND	
NPP	1.7 (43)	ND	

[a] ND, not determined.

RESULTS

Substrate Specificity and Mg Dependence. The nigericin-stimulated nucleoside phosphatase of the sealed microsomal vesicles hydrolyzed specifically ATP and partially GTP (Table II). Other nucleoside triphosphates or diphosphate were hydrolyzed at a slow rate similar to the hydrolysis of *p*-nitrophenol phosphate. Nigericin-stimulated ATP hydrolysis required divalent cations, specifically Mg (Table II) or Mn (data not shown). In the absence of Mg, the rate of ATP and *p*-nitrophenol phosphate hydrolysis were similar. These results indicate Mg-ATP is the true substrate and that 20% to 30% of the total KCl-Mg,ATPase activity (in the presence of nigericin) is due to unspecific substrate hydrolysis. The source of this unspecific phosphatase (identified by Pi release in the absence of Mg) is unclear. The dependence of Mg-ATPase and KCl-Mg,ATPase activity on Mg-ATP concentration is described by a hyperbolic curve with a K_m value of 0.5 mM (28). Thus, the reaction mixture in all subsequent experiments consisted of 3 mM ATP-Tris and 3 mM $MgSO_4$.

pH Dependence. Mg,ATPase activity was characterized consistently by two pH optima: at pH 7.0 and pH 8.0 (Fig. 1a). This result is not surprising since microsomal membranes have different types of Mg-ATPases (13). Nigericin had no effect on Mg-ATPase

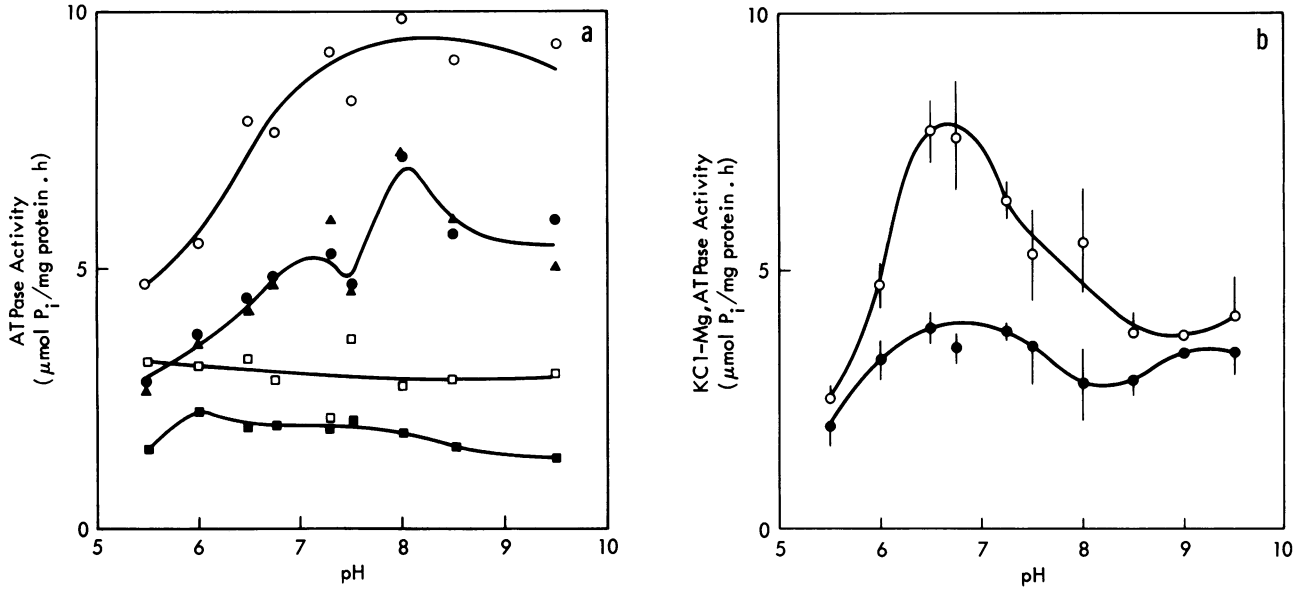

FIG. 1. Effect of pH on ATPase activity in sealed microsomal vesicles from tobacco callus. a, ATPase activity was measured in 30 mM Hepes-Tris buffer at various pH values and 3 mM ATP-Tris in absence of ions (■ or condition A as described in Table I); or in presence of 50 mM KCl (□ or B); 3 mM $MgSO_4$ (● or C); 3 mM $MgSO_4$ plus 5 μM nigericin (▲ or E); or 3 mM $MgSO_4$ and 50 mM KCl (○ or D). b, KCl-stimulated Mg^{2+}-requiring ATPase activity with (○ or ΔC) or without (○ or ΔB) 5 μM nigericin. Results are means ± SEM of three to seven experiments.

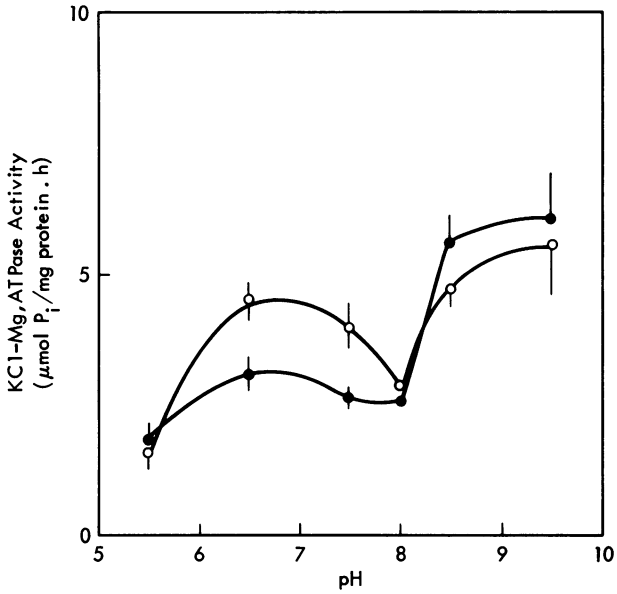

FIG. 2. Effect of pH and nigericin on KCl-Mg,ATPase activity of mitochondrial fraction (500–8,000*g* pellet) from tobacco callus. KCl-Mg,ATPase activity was assayed in absence (●) or presence (○) of nigericin (conditions for ΔB or ΔC as described in Table I). Results are means ± SEM of three to nine experiments.

activity probably because of the absence of counterions for H^+. Unspecific phosphatase activites were not dependent on pH.

KCl-Mg,ATPase activity was optimal around pH 6.5 to 7.0 similar to the pH optimum of K^+-ATPase from oat root plasma membrane (14). Nigericin stimulated KCl-Mg,ATPase by approx. 2-fold at pH 6.75 (Fig. 1b). Optimal ionophore stimulation at this pH is not due to the properties of nigericin because gramicidin D had the same effect (not shown). Some ionophore-stimulated ATPase activity was frequently detected at pH 8.0. ATPase assays were mostly conducted at pH 6.7.

The mitochondrial fraction of autonomous tobacco callus showed optimum KCl-Mg,ATPase activity at pH 8.5 to 9.5 (Fig. 2) as in other plant tissues (9). However, nigericin did not stimulate mitochondrial ATPase activity above pH 8. The reason for this is unclear. Nigericin stimulation of ATPase activity around pH 7 suggested that a population of nonmitochondrial vesicles similar to the sealed microsomal membranes had co-sedimented with the mitochondria. The buoyant densities of plant plasma membranes (1.13–1.18 g/cc) and mitochondria (1.18 g/cc) are relatively close so that a significant fraction of plasma membranes could have sedimented at 13,000*g* along with most of the mitochondria. The two pH optima seen at 6.5 to 7.0 and 9.0 would correspond to the pH optimum of plasma membrane and mitochondrial ATPases, respectively (Fig. 2).

Cation and Anion Sensitivity. Cation sensitivity of the ATPase was investigated by studying the effect of various monovalent cation-chlorides on ATPase activity using gramicidin D. Gramicidin was used instead of nigericin because gramicidin will increase conductance to H^+ and various alkali cations as a channel former (24). Nigericin is a mobile carrier and will exchange H^+ only for alkali-cations in an electroneutral fashion.

In the absence of gramicidin, the ATPase activity was stimulated by cations with a specificity of $NH_4^+ \gg K^+$, Rb^+, Cs^+, Na^+, Li^+ > choline, Tris (Table III). No specificity among the alkali-cations was detected. However, KCl-Mg,ATPase activity was consistently higher than Tris-Cl-Mg,ATPase activity in tobacco callus as well as oat roots (not shown). Therefore, the enzyme activity was more sensitive to alkali cations than unspecific organic cations.

In the presence of gramicidin, ATPase activity was stimulated by cations with an apparent specificity sequence of $NH_4^+ > K^+$, Rb^+, Cs^+, Na^+, $choline^+ > Li^+$. In general, gramicidin stimulated the ATPase activity by 47% to 61% with KCl, NaCl, RbCl, or CsCl similar to nigericin stimulation of KCl-Mg,ATPase (Table III; Ref. 27). LiCl-stimulated Mg,ATPase activity was less sensitive to the ionophore probably because gramicidin-formed channels have poor specificity for Li^+ (24). Gramicidin stimulated choline-Cl-Mg,ATPase activity, whereas nigericin had no effect on Tris-Cl-Mg,ATPase activity (Table III). Inasmuch as these ionophores do not increase conductance to organic ions, the differences observed could be explained by the different modes of ionophore action (24). Gramicidin, but not nigericin, could dissipate H^+ gradients in the absence of K^+. Thus, in the presence of gramicidin, there is no apparent sensitivity to cations.

NH_4^+ is considered independently of the other monovalent

Table III. *Effect of Cation-Chloride on Low-Density Microsomal ATPase Activity with or without Gramicidin*

ATPase activity dependent on 50 mM cation-Cl was measured with 30 mM Hepes-Tris at pH 6.7, 3 mM $MgSO_4$, and 3 mM ATP-Tris with 0.5% ethanol or 2.5 μg/ml gramicidin in 0.5% ethanol. Gramicidin had no effect on Mg-ATPase activity in five of six experiments. Parentheses indicate the number of experiments. Errors are SE.

Cation-Cl	Cation-Cl Mg,ATPase Activity			Gramicidin Stimulation
	−Gramicidin	+Gramicidin	ΔGramicidin	
	μmol Pi/mg protein · h			%
KCl	3.4 ± 0.4	5.3 ± 0.5 (7)	1.9	58
RbCl	3.2 ± 0.4	5.2 ± 0.2 (4)	2.0	60
CsCl	3.6 ± 0.4	5.3 ± 0.6 (4)	1.7	47
NaCl	3.7 ± 0.2	5.9 ± 0.4 (4)	2.2	61
LiCl	3.4 ± 0.4	4.4 ± 0.9 (4)	1.0	28
Choline-Cl	2.7 ± 0.5	5.3 ± 0.6 (3)	2.6	94
Tris-Cl	2.7	2.9[a] (2)	0.2	8
NH_4Cl	5.3 ± 0.8	6.2 ± 0.9 (4)	0.9	17

[a] In the presence of nigericin (2 μM).

Table IV. *Anion Sensitivity of ATPase Activity of Sealed Microsomal Vesicles with or without Nigericin*

K-anion stimulated Mg,ATPase activity was measured with 30 mM Hepes-Tris at pH 6.7 or 8.5, 3 mM $MgSO_4$, 3 mM ATP-Tris with or without 50 mM K-anion. Nigericin = 2 μM. Parentheses indicate the number of experiments. Errors are SE. Using 2.5 μM gramicidin, the ionophore-stimulated ATPase activities in the presence of 50 mM KCl and 25 mM K_2SO_4 were 3.4 and 2.2 μmol Pi/mg protein · h, respectively (in one experiment).

K-Anion	K^+-Anion Mg,ATPase Activity			
	−Nigericin[a]	+Nigericin[a]	ΔNigericin[a]	−Nigericin[b]
	μmol Pi/mg protein · h			
KCl	4.5 ± 0.7	7.4 ± 1.1 (7)	2.9	2.8 ± 0.6 (4)
K-acetate	4.5 ± 0.8	7.4 ± 0.9 (6)	2.9	2.3 ± 0.5 (3)
K_2SO_4	3.0 ± 0.8	5.0 ± 0.7 (6)	2.0	2.3 ± 0.2 (3)
K-benzene sulfonate	1.0	1.5 (2)	0.5	ND[c]
KNO_3	0.1 ± 0.6	0.8 ± 0.5 (6)	0.7	−2.6 ± 1.2 (4)

[a] pH 6.7.
[b] pH 8.5.
[c] ND, not determined.

cations because of its unique property. NH_4^+ can act as an uncoupler by dissipating a pH gradient (data not shown); thus, it is not surprising that NH_4Cl-Mg,ATPase activity was high in the absence of gramicidin (Table III). The ionophore, which can increase conductance to NH_4^+ (24), slightly stimulated ATPase activity.

ATPase activity showed distinct sensitivity to anions as presented in a preliminary report (5). At pH 6.7, Mg-dependent ATPase activity was stimulated by anions with a decreasing selectivity order of $Cl^- = acetate^- > SO_4^{2-} >$ benzene sulfonate$^- > NO_3^-$ both in the presence or absence of nigericin (Table IV). Similar results were seen with gramicidin. Gramicidin-stimulated ATPase activity was 55% higher in the presence of KCl than K_2SO_4. At pH 8.5, the Mg-ATPase activity was inhibited by NO_3^- and slightly stimulated by other anions.

These results could be caused, in part, by dissipation of electrochemical gradients or a direct effect on the enzyme by specific cations or anions or both as explained in "Discussion." Since sensitivity to both cations and anions were observed, the ATPase is referred to as a Mg, salt-stimulated ATPase or Mg,KCl-ATPase for simplicity.

Kinetics of KCl and K_2SO_4 Activation. The concentration of KCl or K_2SO_4 required for half-maximal activation of ATPase activity was not altered by the presence of nigericin. The apparent K_a for KCl-activated ATPase activity was 15.4 and 14.3 mM in the absence and presence of nigericin analyzed from data of Sze (27). K_2SO_4 activated the ATPase activity with an apparent K_a of 7.2 and 7.7 mM in the absence and presence of nigericin (Fig. 3). Thus, the apparent K_a of K^+ alone for both KCl- and K_2SO_4-activated Mg,ATPase activities were similar. These results support the idea that the salt-stimulated ATP hydrolysis in the presence or absence of nigericin is catalyzed by the same enzyme(s). Furthermore, the similarity of the kinetics of K^+ activation indicate that the ATPase is sensitive to cations.

Inhibitors. KCl-Mg,ATPase activity from the sealed microsomal vesicles in the presence of nigericin is sensitive to DCCD, DES, and vanadate but not to oligomycin and azide (Table V). Fusicoccin, which stimulates H^+ extrusion in intact cells (20), had no detectable effect on ATPase activity in the absence or presence of nigericin.

KCl-Mg,ATPase activities were inhibited by increasing concentrations of DCCD (a possible proton-channel blocker) (17) (Fig. 4). The activity of unspecific KCl-stimulated ATP hydrolysis was insensitive to DCCD. Thus, at concentrations greater than 10 μM DCCD, the ATPase activity insensitive to the inhibitor was probably catalyzed by unspecific phosphatases. Using this assumption, the apparent half-maximal inhibition (K_i) by DCCD of KCl-Mg,ATPase activity both in the presence or absence of nigericin was approx. 2 to 3 μM (or 80–120 nmol/mg of membrane protein).

Water-soluble carbodiimides, such as EDAC and CMC, did not inhibit KCl-Mg,ATPase activity (Table VI). The high potency of the hydrophobic carbodiimide (DCCD) indicates that the reactive site is located within a nonpolar region of the membrane. These results could be caused by a covalent reaction of carbodiimides with carboxylate groups of acidic amino-acids involved in proton or cation transport (17).

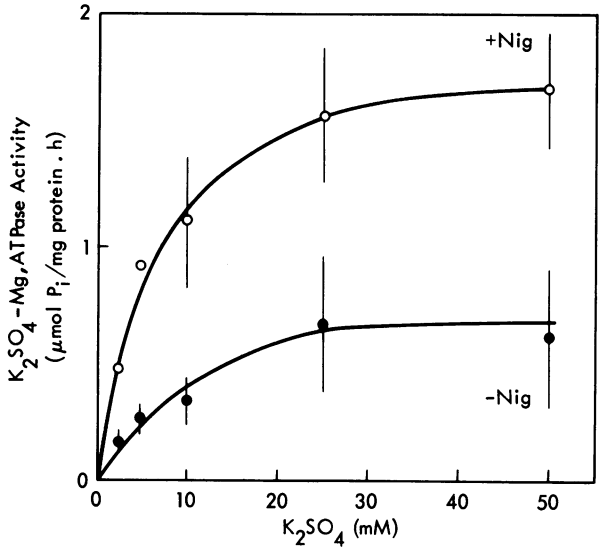

FIG. 3. Kinetics of K_2SO_4 concentration on ATPase activation of sealed microsomal vesicles from tobacco callus. Reaction mixture contained 30 mM Hepes-bis-Tris-propane at pH 6.7, 3 mM $MgSO_4$, 3 mM ATP-Tris with 0.5% ethanol (●), or 2 μM nigericin (Nig) (○) in a final ethanol concentration of 0.5%. Average KCl-Mg,ATPase activities were 1.9 and 4.5 μmol Pi/mg protein · h in absence and presence of nigericin, respectively. Results are average of two experiments. Vertical bars show deviation.

Table V. *Effect of Various Inhibitors on KCl-Mg,ATPase Activity of the Sealed Microsomal and Mitochondrial Fractions from Tobacco Callus*

ATPase activity of the sealed microsomal and mitochondrial fractions was measured at pH 6.7 and 8.5, respectively. Oligomycin (5 μg/ml) = 166 μg/mg microsome protein or 125 μg/mg mitochondria membrane protein. DCCD (10 μM) = 400 nmol/mg microsome protein or 167 nmol/mg mitochondria protein.

Additions	KCl-Mg,ATPase Activity		
	Sealed microsome		Mitochondria
	−Nigericin	+Nigericin	−Nigericin
	% (μmol Pi/mg protein · h)		
None	100 (5.0)	100 (9.9)	100 (7.1)
DCCD (10 μM)	52	28	66
DES (50 μM)	96	63	111
Vanadate (50 μM)	63	66	92
Oligomycin (5 μg/ml)	102	113	55
Azide (1 mM)	122	95	36
DIDS (50 μM)	25	18	ND[a]
Tributyltin (5 μM)	25	ND	ND
Fusicoccin (10 μM)	94	96	113
NH_4 molybdate (0.2 mM)	88	103	ND

[a] ND, not determined.

Both Mg-independent and Mg-dependent ATP hydrolysis were partially sensitive to vanadate (Fig. 5a), making analysis of the vanadate concentration required for half-maximal inhibition difficult. Lack of inhibition of ATPase activity at high vanadate concentrations could be for several reasons. One simple explanation is that ATP hydrolysis at high vanadate concentrations (200–800 μM) was catalyzed by a vanadate-insensitive enzyme. For example, 60% (5 μmol Pi/mg protein · h) of the total Mg-ATPase activity (8.5 μmol Pi/mg protein · h) was vanadate insensitive. ATP hydrolysis insensitive to vanadate was not inhibited by 0.2 mM NH_4-molybdate (Table V) which inhibited unspecific phosphatases (11). According to this model, the apparent K_i of the vanadate-sensitive ATPase in the presence or absence of Mg was approx. 5 μM or less. However, the K_i of vanadate-sensitive KCl-stimulated Mg,ATPase in the presence or absence of nigericin was approx. 30 μM or higher (Fig. 5b). These results

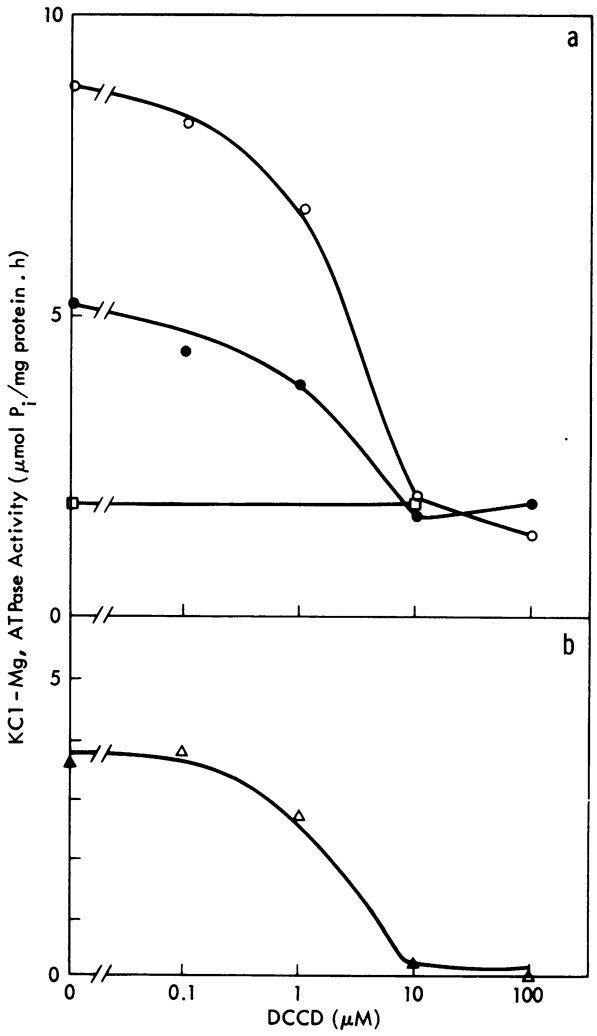

FIG. 4. Inhibition by DCCD of KCl-Mg,ATPase activity of sealed microsomal vesicles from tobacco callus. a, KCl-dependent Mg,ATPase activity (ΔB or ΔC as in Table I) was determined with 30 mM Hepes-Tris at pH 6.5, 3 mM ATP-Tris, 3 mM $MgSO_4$, 50 mM KCl, and 0.5% ethanol with (○) or without (●) 5 μM nigericin (Nig). Unspecific KCl-ATPase activity (B) was measured as above in absence of $MgSO_4$ (□). b, DCCD inhibition of nigericin-stimulated ATPase activity (Δ or ΔD). Results are average of two experiments.

Table VI. *Effect of Various Carbodiimides on KCl-Mg,ATPase Activity of Sealed Microsomal Vesicles from Tobacco Callus.*

Additions		KCl-Mg,ATPase Activity	
		−Nigicerin	+Nigicerin
	μM[a]	μmol Pi/mg protein · h (%)	
None		5.1 (100)	8.8 (100)
DCCD	10	1.9 (38)	2.1 (24)
EDAC	10	5.2 (102)	9.5 (108)
CMC	10	5.2 (102)	8.6 (98)

[a] Concentration of 10 μM = 400 nmol/mg membrane protein.

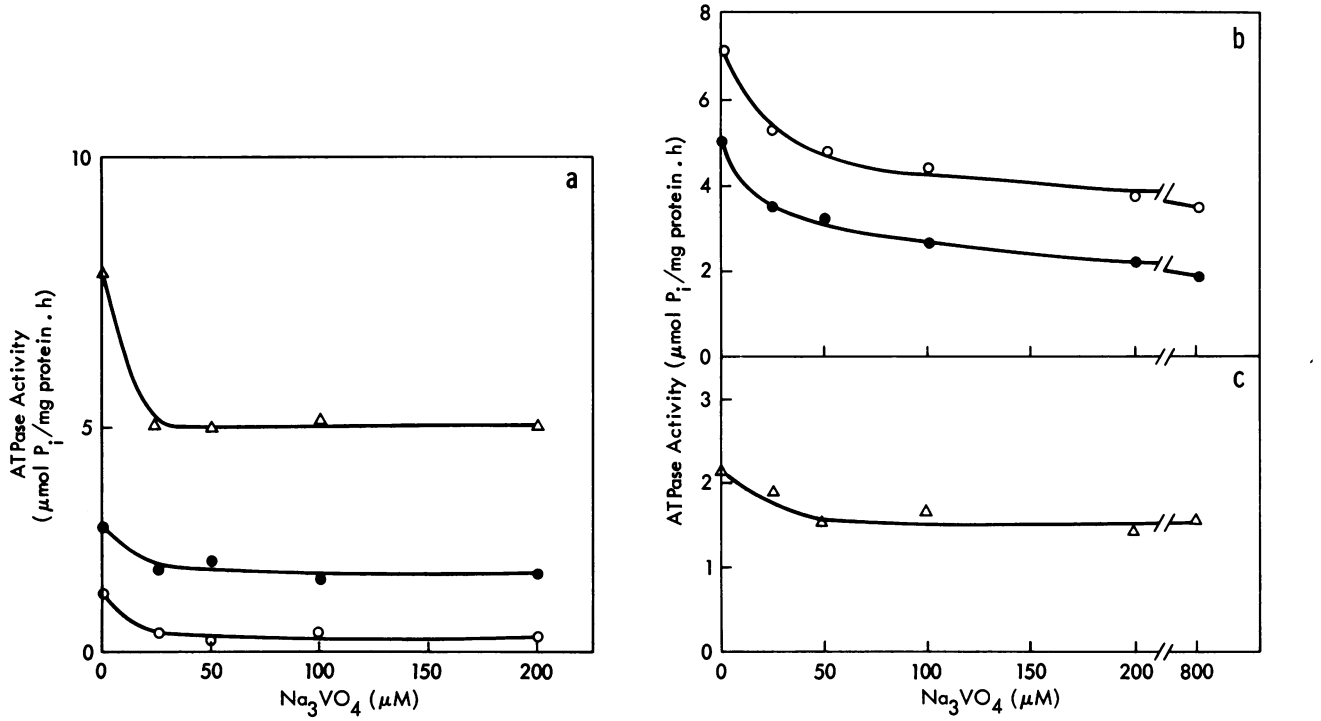

FIG. 5. Partial inhibition by vanadate of ATPase activities of sealed microsomal vesicles from tobacco callus. a, Total ATPase activity at pH 6.7 with no salt (● or A); 3 mM $MgSO_4$ (Δ or C); or KCl-stimulated ATPase in the presence of 50 mM KCl alone (○ or ΔA). b, KCl-Mg,ATPase activity in absence (●) or presence (○) of 5 μM nigericin (ΔB or ΔC). c, Nigericin-stimulated ATPase activity (Δ or ΔD). Results are average of two experiments. (Capital letters refer to conditions described in Table I).

suggest that KCl might alter the sensitivity of the enzyme to vanadate or that KCl-stimulated Mg-dependent ATP hydrolysis and most of the Mg-dependent ATP hydrolysis were catalyzed by two different enzymes. Whatever the reason might be, approx. 40% of the total KCl-Mg,ATPase activity in the absence of nigericin and 70% of the nigericin-dependent KCl-Mg,ATPase activity was vanadate insensitive (Fig. 5c). The possibility of two KCl-stimulated Mg,ATPases, one vanadate sensitive and one vanadate insensitive, could be better understood after separation and purification of the membrane-associated enzyme(s).

DIDS, recently shown to be a specific anion transport inhibitor in corn protoplasts (16), was not a specific ATPase inhibitor. ATPase activity of the mitochondria (data from oat roots are not shown) and sealed microsomal vesicles were inhibited (Table V). This finding is not surprising. Besides inhibiting the amino-groups of amino acids involved in anion transport, DIDS may have access in isolated membranes to other amino-groups required for ATPase activity. These results, nevertheless, could also suggest that ATPase activity depends on the rate of anion transport. Tributyltin, which inhibits ATP synthetase systems and can exchange Cl^- for OH^- (17), inhibited ATPase activity in the absence or presence of KCl (Table V).

DISCUSSION

ATPase activity stimulated by nigericin, gramicidin, or CCCP in combination with valinomycin, was interpreted as evidence that the ATPase of sealed microsomal vesicles mediated a H^+/K^+ exchange transport (27), but the results provide no information about how H^+, K^+, or anions move and which membranes have ion-pumping ATPases (27, 28). This study attempts to clarify some of these questions and to relate the ionophore-stimulated ATPase to other well-studied ATPases. *In vitro* studies showing ATP-dependent generation of an electrical potential (inside positive) (28) and a pH gradient (inside acid) (6, 7, 21, 26, 28) would suggest that the ionophore-stimulated ATPase(s) are H^+-pumping ATPase(s). However, the possibility that other ions (including cation, anion, or both) are also translocated by ATPases cannot be excluded yet. Thus, the term 'nigericin-stimulated ATPase' (or ionophore-stimulated ATPase) will be broadly used to include H^+ and ion-pumping ATPases.

Similarity of Nigericin-Stimulated ATPase and Mg, KCl-ATPase. The findings in this and previous papers show that the nigericin-stimulated ATPase activity and the KCl-Mg,ATPase activity associated with nonmitochondrial membranes were catalyzed by the same or similar enzymes. The following properties of KCl-Mg, ATPase in the presence or absence of nigericin would support this idea: (a) cell fractionation studies show that the distribution of total activities of both enzyme activities in various subcellular fractions was similar (27). (b) Both enzyme activities required Mg^{2+} and ATP (Table II). (c) The pH optimum of both enzyme activities was approx. 6.5 to 7.0 (Fig. 1). (d) Concentration kinetics of KCl or K_2SO_4 activation of the ATPase activity was similar in the absence or presence of nigericin (Ref. 27; Fig. 3). (e) Both enzyme activities were insensitive to mitochondrial ATPase inhibitors and sensitive to inhibitors of the plasma membrane ATPase, such as vanadate, DCCD, and DES (Refs. 27, 28; Table V).

The plasma membrane ATPase from several plant tissues (8, 9, 14, 22) have properties similar to the nigericin-stimulated ATPase from tobacco callus. The results would support the conclusion that the ionophore-stimulated enzyme activity is catalyzed, in part, by a KCl-Mg,ATPase of the plasma membrane (27, 28). However, some properties of the nigericin-stimulated ATPases (anion-sensitivity and partial vanadate insensitivity) would suggest involvement of other enzymes.

Anion and Cation Sensitivity. Cation and anion sensitivities of ATPase (Tables III and IV) could be caused by one or a combination of several of the following possibilities: (a) direct transport of cations or anions, (b) direct and specific ionic effects on the enzyme; (c) lipid-permeable properties of the ions and their ability to dissipate pH gradient or membrane potential, and (d) unspecific

ionic strength effects. There is evidence mainly for the second and third possibilities. Experiments are underway to distinguish among all the possibilities.

ATPase sensitivity to cations, although small, was supported by the following observations: (a) the kinetics of K^+ activation by KCl or K_2SO_4 were similar (Fig. 3; Ref. 27) and (b) K^+ stimulated ATPase activity more than $Tris^+$ (Table III). Lack of selectivity between the alkali-cations was surprising, since plasma membrane ATPase from oat roots or corn leaves is selective for K^+ (22, 29). Perhaps, cation specificity is difficult to detect in sealed vesicles that are more sensitive to anions.

ATPase sensitivity to anions is illustrated by the following: (a) specific stimulation of ATPase activity by Cl^- over other inorganic anions in the presence or absence of ionophores (nigericin or gramicidin); (b) inhibition of ATPase activity by NO_3^- (Table IV); and (c) inhibition of KCl-Mg,ATPase activity by DIDS, an anion transport inhibitor (Table V). Anion stimulation of ATPase activity can be partly explained by dissipation of a membrane potential by permeant anions. Chloride, but not sulfate, dissipated a membrane potential (interior positive) generated by ATP in sealed microsomal vesicles of tobacco callus (28). Cl^- also appears to stimulate the enzyme specifically since enzyme activity is optimal with Cl^- even in the absence of electrochemical gradients (induced by gramicidin). Inhibition of ATPase activity by KNO_3 and DIDS would suggest that an anion (*e.g.* Cl^-) is specifically transported via an anion channel into the vesicles (5) as proposed by Hager and Helmle (7). It is not clear how organic anions (*e.g.* acetate) stimulate ATPase activity.

Enrichment of ATPase activity more sensitive to anions than cations in the sealed microsomal vesicles could be caused by one enzyme but is probably catalyzed by several ion-pumping ATPases. Gramicidin stimulation of ATPase activity in the absence of K^+ (Table III) suggests that H^+ is the major ion pumped. ATP-dependent pH gradient formation in the vesicles (interior acid) is consistent with this conclusion (6, 7, 21, 26, 28). However, specific stimulation by Cl^- and the small sensitivity to alkali-cations of the ATPase activity would suggest that primary or secondary transport of anions and cations need to be considered.

Types of Nigericin-Stimulated ATPases. Studies with vanadate suggest that plant membranes may have at least two classes of nigericin-stimulated ATPases: one sensitive and one insensitive to vanadate (Fig. 5). Vanadate inhibits many phosphohydrolases that have a covalent phosphoenzyme intermediate in their mechanism of action (19). The vanadate competes with phosphate for binding and is highly effective as an inhibitor possibly because the pentavalent vanadium can easily adopt a stable trigonal bipyramidal structure that resembles the transition state of the phosphate during reaction. A vanadate-insensitive ATPase could be interpreted as an enzyme that does not form a covalent phosphoenzyme intermediate. In *Neurospora*, vanadate completely inhibits the plasma membrane ATPase activity at 50 μM (2). However, 200 μM vanadate only partially inhibited the KCl-Mg,ATPase activity of the low-density microsomal fraction from tobacco callus (Fig. 5). Plasma membrane Mg^{2+},K^+-ATPase from corn leaves showed similar kinetics of partial inhibition by vanadate (22). Perlin and Spanswick (22) suggested that a nonspecific phosphatase was vanadate-insensitive, however, nonspecific phosphatases alone could not account for all the inhibitor-insensitive activity (Fig. 5). Sze and Churchill (28) showed that 200 μM vanadate inhibited 60% of the ATP-dependent SCN^- uptake (membrane potential generation) in sealed microsomal vesicles. However, ATP-dependent methylamine uptake (pH gradient generation) was unaffected by 200 μM vanadate (K. A. Churchill and H. Sze, unpublished results) similar to reports recently published (6, 7). Partial inhibition of ATPase activity by high vanadate concentrations indicate that the low-density vesicles contain at least two classes of electrogenic proton pumps.

Identity of Membrane Vesicles. It was concluded in previous studies that the ionophore-stimulated ATPase originated in part from inside-out plasma membrane vesicles and perhaps, in part from right-side-out tonoplast vesicles (27, 28). The present study would support this idea. Some of the properties of the ionophore-stimulated ATPases are similar to an ATPase associated with red beet tonoplast (12, 30), yeast vacuolar membrane (11), a microsomal membrane fraction equilibrating in sucrose gradients at approx. 1.13 g/cc (3, 8) or approx. 32% sucrose (7). In these studies, an ATPase was sensitive to anions with Cl^- being the most stimulatory and insensitive to cations (3). The anion-stimulation of the enzyme was most distinct at pH 8.0 to 8.5 (3, 8, 12) and inhibited by NO_3^- (3, 8, 30) similar to an anion-sensitive ATPase postulated to be on the tonoplast (8, 25). The enzyme from the red beet tonoplast is vanadate-insensitive (30), similar to a H^+-pumping ATPase found in yeast vacuolar membrane (11) and in a low-density microsomal fraction from maize coleoptile (7). The parallel properties between ATPases of the published studies and this work would suggest that part of the ionophore-stimulated ATPase activity could be attributed to enzymes on tonoplast vesicles. However, I do not know whether the vanadate-sensitive and vanadate-insensitive ATPases are localized preferentially on the plasma membrane and tonoplast, respectively, or whether both classes of enzymes are found on the same membranes. Because membrane synthesis may originate from ER and Golgi, the presence of ion-pumping ATPases on these membranes is also considered.

In summary, at least two classes of nigericin-stimulated ATPases are associated with nonmitochondrial membranes: one vanadate-sensitive and one vanadate-insensitive. The results suggest that plant membranes have at least two different classes of electrogenic ATPases acting as H^+ or ion pumps. Properties of the nigericin-stimulated ATPases would suggest that the ion-pumping ATPases originated in part from the plasma membrane and in part from other nonmitochondrial membranes, such as the tonoplast.

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