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Ecto-enzymes of mammary gland and its tumours

Ca^{2+}- OR Mg^{2+}-STIMULATED ADENOSINE TRIPHOSPHATASE AND ITS PERTURBATION BY CONCANAVALIN A

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Intact viable 13762 mammary-adenocarcinoma ascites cells hydrolyse added ATP. The localization of hydrolysis product and inactivation by the slowly penetrating chemical reagent diazotized sulphanilic acid indicate that this ATPase is at the external surface of the cell. A number of features differentiate this enzyme from mitochondrial, myosin and cation-transport ATPases. It is stimulated by either Ca^{2+} or Mg^{2+} and has little or no activity in their absence. It is insensitive to ouabain, oligomycin and azide. It is the major ATPase activity found in homogenates of gently disrupted 13762 cells. The ATPase activity is inhibited at high substrate concentrations and shows an apparent stimulation by concanavalin A in isolated membranes, but not in intact cells. The stimulation by concanavalin A results predominantly from a release from substrate inhibition.

Cell-surface enzymes are potentially useful markers of membrane changes which occur as a result of cell differentiation, various disease states or artificial perturbations (Stefanovic *et al.*, 1976; Carraway *et al.*, 1976, 1979). A problem with the use of marker enzymes is the lack of information on the properties of these enzymes and their physiological functions. For example, an ecto-ATPase activity (Trams & Lauter, 1974; DePierre & Karnovsky, 1974*a*,*b*; Ronquist & Ågren, 1975; Stefanovic *et al.*, 1976) has been described in a number of cell types. Its external location suggests that it is different from previously described ATPases, which are found at the interior surface of plasma membranes and often supply the energy requirement for transport functions. In the human erythrocyte membrane, the best-studied membrane model, there are at least three ATPases (Drickamer, 1975). The ouabain-sensitive enzyme is involved in Na^+ and K^+ transport and requires Mg^{2+}. A second enzyme is involved in Ca^{2+} transport and requires Mg^{2+} for activity. The third enzyme is designated Mg^{2+}-ATPase, and no function has been ascribed to it. In some more complex cells an ATPase activity has been described which is stimulated by either Mg^{2+} or Ca^{2+} (Parkinson & Radde, 1971). Ehrlich ascites-tumour cells (Ronquist & Ågren, 1975) and some neural cells (Stefanovic *et al.*, 1976) hydrolyse exogenously added ATP by such an enzyme. Several previous studies have established the existence of ecto-ATPases which are stimulated by Mg^{2+} (DePierre & Karnovsky, 1974*a*,*b*; Trams & Lauter, 1974). Whether these activities are also stimulated by Ca^{2+} has not been reported.

In the present study we have used established criteria (DePierre & Karnovsky, 1974*a*) to demonstrate the presence at the cell surface of 13762 mammary-adenocarcinoma cells of an ATPase activity which is stimulated by either Mg^{2+} or Ca^{2+}. The enzyme in isolated membranes shows an apparent activation on treatment with concanavalin A. However, this activation is due largely to release from substrate inhibition at high ATP concentrations. Intact cells show neither concanavalin A activation nor substrate inhibition.

Experimental

Materials

ATP, GTP, ADP, glucose 6-phosphate, oligomycin, iodoacetic acid and ouabain were obtained from Sigma, as were buffers and salts, all reagent grade. *p*-Nitrophenyl phosphate was supplied by Nutritional Biochemicals Corp., sulphanilic acid by Fisher Scientific Co. and concanavalin A by Miles Laboratories [γ-^{32}P]ATP was from Amersham.

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Isolation of cells and membranes

The 13762 MAT-A and MAT-C1 mammary ascites adenocarcinomas were maintained in Fischer 344-strain female rats. Cells and Zn^{2+}-stabilized membrane envelopes of the cells were obtained as previously described (Carraway *et al.*, 1976, 1979). Membranes were stored frozen for no longer than 3 days before enzyme studies and were frozen and thawed no more than once, since the ATPase appears somewhat labile.

Enzyme assays

Ca^{2+}- or Mg^{2+}-activated ATPase was assayed by incubating cells (approx. 10^6 per assay) or membranes (approx. 50μg of protein) at 37°C for 2–10min in 25mM-histidine/25mM-imidazole/120mM-KCl/1mM-ouabain (pH 8.2) and the appropriate concentration of ATP and Ca^{2+} or Mg^{2+} (usually 5mM bivalent cation) in a 0.4ml assay volume. Both unlabelled ATP and [γ-^{32}P]ATP were used as substrates. The reaction was terminated by addition of 0.5ml of 10% (w/v) trichloroacetic acid. Samples chilled on ice for 10min were centrifuged and samples taken for assay of P_i by the method of Chen *et al.* (1956) or by scintillation counting after treatment with charcoal to remove nucleotides (Doss *et al.*, 1979). Controls containing no enzyme were used to correct for non-enzymic hydrolysis. The same procedure was used in assays with GTP, ADP, glucose 6-phosphate and *p*-nitrophenyl phosphate as substrate, by using unlabelled compounds and chemical analyses. Ouabain-sensitive ATPase (Shin & Carraway, 1973), lactate dehydrogenase (Neilands, 1955) and 5′-nucleotidase (Carraway *et al.*, 1976) were assayed as previously described.

Other assays

Protein was measured by the procedure of Lowry *et al.* (1951). Cells were quantified by counting in a haemocytometer.

Treatments with diazotized sulphanilic acid

Diazotized sulphanilic acid was prepared by the procedure of DePierre & Karnovsky (1974*a*). Treatments with it before and after cell homogenization were performed as follows.

(1) *Treatment of intact cells.* Washed intact cells (5.3×10^7) were treated for 15min at 37°C with 1.0mM- or 5.0mM-diazotized sulphanilic acid in 2.0ml of iso-osmotic (10mM) Hepes [4-(2)-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/saline buffer, pH 7.4. The treated cells were washed twice in Hepes; samples were taken for assays of Ca^{2+}- or Mg^{2+}-ATPase and lactate dehydrogenase. Another sample of treated cells was homogenized at 0°C in 40mM-Tris/HCl, pH 7.4, by five strokes of a Dounce B homogenizer. Over 99% of the cells were broken by this procedure. The homogenate was assayed for Ca^{2+}- or Mg^{2+}-ATPase and lactate dehydrogenase (Neilands, 1955).

(2) *Treatment of homogenate.* Washed intact cells were homogenized in the same manner and samples of the homogenate subsequently treated with 1.0mM- and 5.0mM-diazotized sulphanilic acid. The treated homogenates were assayed for Ca^{2+}- or Mg^{2+}-ATPase and lactate dehydrogenase.

Results

Cell-surface localization of ATPase

To evaluate the relationship between the ATPase and other cell-surface properties, two different ascites sublines of the 13762 rat mammary adenocarcinoma, MAT-A and MAT-C1, were used. As noted previously (Carraway *et al.*, 1979), these sublines differ markedly in morphology and concanavalin A-receptor mobility. However, no significant differences between the sublines were found in the properties of the ATPase. Similar results were obtained previously for 5′-nucleotidase (Carraway *et al.*, 1979). The experimental values reported here are averages of duplicate determinations, except as noted, varying less than 15% from the reported value. The major source of variation was in the cell and membrane preparations, resulting in different specific activities. Moreover, each type of experiment has been repeated on the other subline for verification of the result. Therefore the presentation below will simply refer to the cells as 13762 MAT cells without regard to the subline used.

To establish that the ATPase of the 13762 cells is an ecto-enzyme, we have used criteria developed by DePierre & Karnovsky (1974*a*). In addition, all ATPase-perturbation studies were performed in the presence of Mg^{2+} and in the presence of Ca^{2+} in parallel experiments to show that this enzyme can be activated by either cation. A number of experimental results indicate the external membrane location of the enzyme. (1) Externally added ATP is hydrolysed by cell populations containing no more than 5% non-viable cells, as measured by Trypan Blue exclusion. This evidence does not rule out the possibility of transient or localized ATP uptake by the cells. However, such an uptake mechanism, if present, does not appear to be energy-dependent, since treatment with azide or iodoacetate as energy poisons does not significantly decrease ATP hydrolysis by the intact cells. (2) Assays of medium in which cells have been incubated show less than 3% of the activity in cells, indicating that ATP hydrolysis is not due to a released enzyme. (3) [^{32}P]ATP was hydrolysed by cells that had been previously loaded with [^{33}P]phosphate (DePierre & Karnovsky, 1974*a*). The distribution of ^{32}P and ^{33}P

in the cells and supernatant indicated that over 90% of the enzyme activity must be extracellular. (4) The ATPase determined in the intact cell represents more than 90% of the ouabain-insensitive, Ca^{2+}-stimulated, and more than 80% of the ouabain-insensitive, Mg^{2+}-stimulated activity of homogenates obtained by gentle homogenization of the cells. Membrane preparations were assayed for Na^+- and K^+-dependent ATPase (Shin & Carraway, 1973) in the presence and absence of ouabain. The ouabain-insensitive ATPase activity represented 60–70% of the total ATPase activity. These results suggest that the enzyme studied in the intact cell is the same as that of the isolated cell-surface membrane and a major contributor to the total ATPase activity of the cells. (5) Cells and homogenates were treated with diazotized sulphanilic acid (DePierre & Karnovsky, 1974*a*), a slowly penetrating chemical reagent which reacts covalently with a number of protein functional groups. Brief treatments of intact 13762 cells with diazotized sulphanilic acid caused marked decreases in the ATPase activity, assayed in either intact cells or homogenates (Table 1). Inactivation of Ca^{2+}- and Mg^{2+}-stimulated activities was essentially the same. The diazotized sulphanilic acid treatment of intact cells had much smaller effects on lactate dehydrogenase, assayed in homogenates. When homogenates were treated with diazotized sulphanilic acid, lactate dehydrogenase was inhibited by over 95%. ATPase activities in homogenates and in intact cells treated with diazotized sulphanilic acid were inhibited almost to the same extent. The results of the chemical modification experiments closely parallel those of DePierre & Karnovsky (1974*a*) on ecto-phosphohydrolases and strongly support the postulate of an external location of the ATPase.

Kinetic properties and inhibitor effects on ATPase activities in intact cells and isolated membrane envelopes

There are several features which distinguish the activities reported here from the more commonly studied ATPases, such as those of the erythrocyte membrane, sarcoplasmic reticulum or mitochondria. The ecto-ATPase is stimulated independently by Ca^{2+} or Mg^{2+} in the absence of the other cation either in the intact cell (Fig. 1) or in isolated membranes (Fig. 2). These membranes are obtained after Zn^{2+} 'stabilization' as envelopes ('ghosts') derived from the cell surface. Phase-contrast microscopy shows a tear in the envelope through which the nucleus and cytoplasm were extruded. Thus both inner and outer surfaces of the membrane

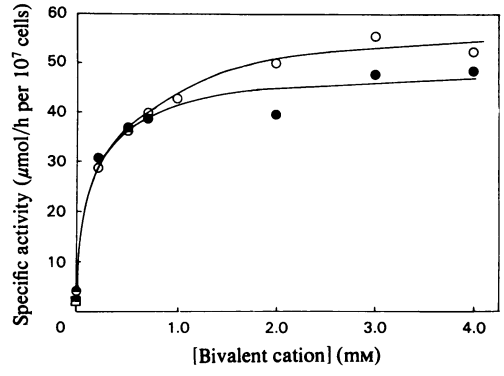

Fig. 1. *Activation of intact 13762-cell ATPase by bivalent cations*

Assays contained 2.5×10^5 cells and 2.5 mM-ATP. For details see the text. Additions: ●, Ca^{2+}; ○, Mg^{2+}; ■, EDTA, □, no bivalent cations.

Table 1. *Effect of diazotized sulphanilic acid (DSA) on ATPase and lactate dehydrogenase activities of whole cells and homogenates*

For details see the text.

Treatment	ATPase (μmol/h per 10^7 cells) Ca^{2+}	ATPase (μmol/h per 10^7 cells) Mg^{2+}	Lactate dehydrogenase (μmol/h per 10^7 cells)
Intact			
Untreated	55	59	3.7
1 mM-DSA	21	23	4.4
5 mM-DSA	3	2	4.8
Cells homogenized before DSA treatment			
Untreated	51	53	690
1 mM-DSA	14	12	410
5 mM-DSA	—	—	27
Cells homogenized after DSA treatment			
1 mM-DSA	15	19	610
5 mM-DSA	3	7	510

Table 2. *ATPase and 5'-nucleotidase activities in cell fractions during plasma-membrane purification*
Values in parentheses represent the degree of purification.

Fraction	Activity (μmol/h per mg of protein)		
	Mg^{2+}-ATPase	Ca^{2+}-ATPase	5'-Nucleotidase
Intact cells	9.5	8.0	1.7
Crude membrane pellet	7.8	6.7	1.6
Purified membranes	48 (5.1)	38 (4.8)	9.9 (5.8)

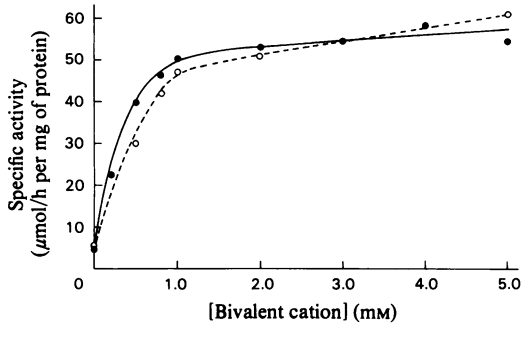

Fig. 2. *Activation of 13762-cell membrane ATPase by bivalent cations*
Assays contained 85 μg of membrane protein for the study with Ca^{2+} (●) and 34 μg for the study with Mg^{2+} (○). ATP concentration was 2 mM.

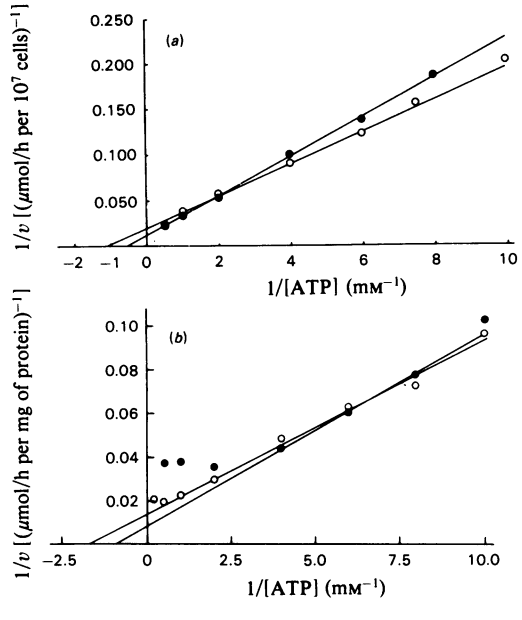

Fig. 3. *Lineweaver–Burk plots of intact 13762-cell (a) and membrane (b) ATPase*
Assays contained 9.2×10^4 cells. For details see the text. ●, Ca^{2+}; ○, Mg^{2+}.

are accessible. The responses of the activity to Ca^{2+} and Mg^{2+} are nearly the same. There is a small amount of residual activity (approx. 3%) in the absence of added bivalent cation, which is not removed by the addition of 1 mM-EDTA. The effects of the Ca^{2+} and Mg^{2+} are additive when they are added at less than saturating concentrations, suggesting that they act at the same site.

Oligomycin does not substantially inhibit the Mg^{2+}- or Ca^{2+}-ATPase activity in intact cells; homogenate enzyme is inhibited by less than 20%.

The fact that no substantial increase of ATPase is observed in breaking the cell permeability barrier in the preparation of homogenates again suggests that this ATPase is the major ATPase activity of these cells. To verify that the membrane and cell-surface activities are the same enzyme, the purification of ATPase was compared with 5'-nucleotidase, which has been established as an ecto-enzyme in these cells (Carraway *et al.*, 1976). There is a close correspondence between the purification of the nucleotidase and Mg^{2+}- or Ca^{2+}-ATPase (Table 2).

The fact that Mg^{2+}- and Ca^{2+}-stimulated activities show parallel behaviour during membrane isolation and inhibition by diazotized sulphanilic acid suggests that they reside on a single enzyme. Kinetic studies on the enzyme support this contention. Fig. 3 shows double-reciprocal plots of enzyme-kinetic data with ATP as substrate for intact cells and membranes. K_m values are 0.9 mM and 0.6 mM for the Mg^{2+}-activated enzyme and 1.8 mM and 1.1 mM for the Ca^{2+}-activated enzyme in the intact cells and membranes respectively. In the isolated membranes substrate inhibition can be seen. This inhibition, which varies with different membrane preparations, is greater in the presence of Ca^{2+} than with Mg^{2+}. Substrate inhibition has not been observed in intact cells. No inhibition with increasing ATP concentration is noted at suboptimal bivalent-cation concentrations (results not shown). Therefore the substrate inhibition observed is not due to free ATP. The results suggest that the substrates and inhibitors for the Ca^{2+}- and Mg^{2+}-activated enzymes are the respective bivalent-cation–ATP complexes, although a complete kinetic analysis has not been performed.

Other phosphohydrolase activities

Intact 13762 cells also show substantial

hydrolytic activity against externally added GTP under ATPase-assay conditions. Although the Ca^{2+}-stimulated activity exhibits simple kinetics (K_m for GTP 3mM), the Mg^{2+}-stimulated GTP hydrolysis shows a biphasic double-reciprocal plot (K_m values 3 and 0.4mM), suggesting two different enzymes. ADP hydrolysis proceeds at about one-fifth the rate of ATP hydrolysis and exhibits complex kinetics. The ATPase activities observed here are not due to non-specific or other phosphatase activities, since the isolated membranes prepared by the Zn^{2+}-stabilization procedure show essentially no hydrolytic activities against glucose 6-phosphate and *p*-nitrophenyl phosphate. The absence of these enzyme activities is not due to inactivation of the enzymes during membrane preparation (Shin & Caraway, 1973).

Concanavalin A perturbation of the membrane ATPase

Our previous studies (Carraway *et al.*, 1975) showed that Mg^{2+}-ATPase of partially purified rat mammary plasma membranes was activated by the plant lectin concanavalin A. Similar experiments with MAT cells and plasma membranes showed rather unusual behaviour. At low or intermediate substrate concentrations (up to 2.5mM-ATP) the enzyme activity was not substantially changed in either cells or membranes. Small degrees of either activation or inhibition (up to about 15%) were observed in different membrane preparations. However, at higher ATP concentrations a definite activation was observed (Table 3). The amount of activation and the concentration of ATP necessary to observe activation were variable with different membrane preparations. Since activation by concanavalin A and substrate inhibition appeared to be correlated in different preparations, the kinetics of the enzyme were examined in the presence and absence of concanavalin A. Fig. 4 shows that the increased ATPase activity is not due to a true activation, but instead results from a relief of substrate inhibition. As observed previously for the mammary membranes, the concanavalin A effect

Table 3. *Effect of concanavalin A on membrane Mg^{2+}-ATPase*

Samples were incubated for 30min at 37°C with concanavalin A before enzyme assays. Values are determinations for a typical membrane preparation.

Conditions		
ATP (mM)	Concanavalin A (μg/ml)	Activity (μmol/h per mg of protein)
2.5	0	6.9
2.5	200	5.9
2.5	500	6.3
5.0	0	4.2
5.0	200	7.8
5.0	500	10.2

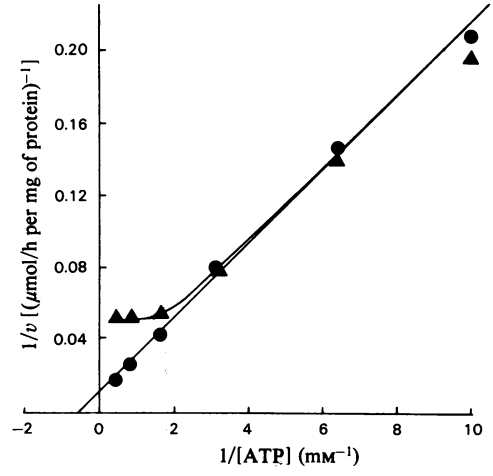

Fig. 4. *Lineweaver–Burk plot of membrane ATPase with and without concanavalin A*

Membranes (43μg of protein/assay) were incubated for 30min at 37°C in assay medium containing 5mM-Mg^{2+} in the absence (▲) and presence (●) of 660μg of concanavalin A/mg of protein (80μg/ml) before addition of substrate.

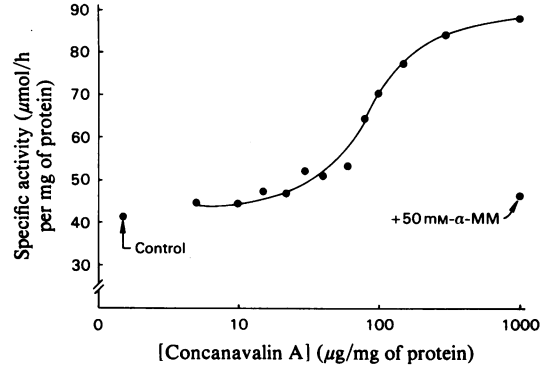

Fig. 5. *Concentration-dependence of concanavalin A stimulation of membrane ATPase*

Membranes (30μg of protein/assay) were incubated in assay medium containing 5mM-Ca^{2+} and concanavalin A [5–1000μg/mg of protein (0.4–89μg/ml)] for 20min at 37°C before assay with 2mM-ATP. The same experiment performed on membrane suspensions containing 5mM-Mg^{2+} gave a very similar activation plot and identical Hill coefficient (1.7). Abbreviation: α-MM, α-methyl mannoside.

(Fig. 5) is concentration-dependent and exhibits positive co-operativity (Hill coefficient 1.7).

In contrast, no activation of the enzyme was observed by treatment of intact cells with concanavalin A at substrate concentrations of 0–5 mM-ATP and concanavalin A concentrations as high as 500 μg/ml.

Discussion

The ATPase described in this study is unique in a number of ways. Its exterior location and lack of response to ouabain, oligomycin and azide distinguish this enzyme from the Na^+,K^+- and Ca^{2+}-ATPases associated with transport of these ions and from the Mg^{2+}-ATPase present in erythrocytes and other cells. The activities activated by Ca^{2+} and by Mg^{2+} show parallel behaviour under essentially all conditions tested. The results are best explained by a single enzyme stimulated by both cations. The response to cations also distinguishes this enzyme from other eukaryotic membrane ATPases and from myosin, which has been postulated to be present at the cell surface (Willingham *et al.*, 1974). The behaviour toward oligomycin, Ca^{2+} and Mg^{2+} appears more similar to some of the bacterial ATPases (Abrams & Smith, 1970) than to the mammalian enzymes. However, many of the bacterial enzymes are also inhibited by azide. The ecto-ATPase represents a major ATP-hydrolysing activity of the 13762 cells and of their isolated plasma membranes.

The presence of an ecto-ATPase immediately raises questions as to its function. An obvious answer is that it serves to control the extracellular ATP concentration. It is more difficult to specify exactly what ATP does at the external surface of cells and how it gets there. ATP alters cell volume and ion fluxes (Rorive & Kleinzeller, 1972), morphology in mast cells (Kruger *et al.*, 1974), cell adhesion, aggregation and movement in fibroblasts (Jones, 1966; Knight *et al.*, 1966) and insulin stimulation of glucose transport in adipocytes (Chang & Cuatrecasas, 1974). External ATP also has an effect on *p*-nitrophenyl phosphate transport which differs in normal and transformed 3T3 cells (Rozengurt & Heppel, 1975). The basis or bases for these ATP effects are unknown, but they may be related to protein kinase activity at cell surfaces (Mastro & Rozengurt, 1976). The source of the extracellular ATP is uncertain, but there is evidence that ATP may be translocated from the cytosol to the cell exterior (Trams, 1974).

The observations on substrate inhibition of the ATPase are intriguing. If substrate inhibition is explained by a two-site model, the fact that substrate inhibition is observed in membranes, but not in cells, may indicate that the second (non-substrate) site is inside the cell. Such an arrangement could permit control of the enzyme activity by the cellular ATP concentration. This control could be over-ridden from outside the cell by factors such as concanavalin A, which interact with carbohydrates at the cell surface. Although the mechanism of action of concanavalin A is not presently clear, it could cause a conformational change in the enzyme which blocked the second ATP site.

Exactly how our results relate to previously described effects of concanavalin A on membrane ATPases (Novogrodsky, 1972; Jarett & Smith, 1974; Luly & Emmelot, 1975; Pommier *et al.*, 1975) is not known. Riordan *et al.* (1977) have described an activation of a Mg^{2+}-ATPase of liver membranes which does not appear to be related to substrate inhibition and which is sensitive to temperature effects on the membrane. Whether this is an ecto-enzyme and is stimulated by Ca^{2+} as well as by Mg^{2+} is unknown. It seems likely that there is more than one mechanism for ATPase stimulation by concanavalin A.

Clearly membrane enzymes as sensitive as ATPases can be altered by a number of membrane perturbants and should be useful in understanding membrane structure–function relationships.

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