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Evidence that Mg^{2+}- or Ca^{2+}-activated adenosine triphosphatase in rat pancreas is a plasma-membrane ecto-enzyme

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Preparations of enzymically dispersed rat pancreatic cells hydrolyse externally added nucleoside triphosphates and diphosphates at high rates in the presence of Mg^{2+} or Ca^{2+}. The lack of response to specific inhibitors and activators differentiates this hydrolytic activity from that of other well-characterized ion-transporting ATPases. Studies based on inactivation of this hydrolytic activity by the covalently reacting, slowly permeating probe diazotized sulphanilic acid indicated that this nucleoside tri- and di-phosphatase is primarily a plasma-membrane ecto-enzyme. It is the major ATPase activity associated with intact cells, homogenates and isolated plasma-membrane fractions. Concanavalin A stimulates this ATPase activity of intact cells and isolated plasma-membrane fractions. The insensitivity of this ATPase activity to univalent ions and inhibitors of pancreatic electrolyte secretion, taken together with the evidence that the active site is externally located, suggests that this enzyme is not directly involved in HCO_3^- secretion in the pancreas. Its actual function remains unknown.

Cells obtained from a variety of tissues have been shown to contain intrinsic plasma-membrane enzymes whose active sites are externally rather than internally orientated (DePierre & Karnovsky, 1973; Trams & Lauter, 1974). Such entities have been termed 'ecto-enzymes' and are of uncertain physiological function. In contrast, the active sites of the well-characterized ion-transporting enzymes such as $(Na^+ + K^+)$-activated ATPase, Ca^{2+}-activated ATPase and the gastric $(H^+ + K^+)$-activated ATPase face the cytoplasm (Schuurmans-Steckhoven & Bonting, 1981).

Recently we described a Mg^{2+}- or Ca^{2+}-activated ATPase (Mg/Ca-ATPase) in plasma-membrane fractions isolated from whole homogenate of rat pancreas (Martin & Senior, 1980). This ATPase was characterized by a very high specific activity (about 30 μmol of ATP hydrolysed/min per mg of protein) and was insensitive to univalent cations, anions, ouabain, vanadate, Ruthenium Red, oligomycin and the specific mitochondrial H^+-translocating ATPase inhibitor protein. This ATPase activity was of interest because of its potential role in the secretion of HCO_3^- by the pancreas. Pancreatic HCO_3^- secretion occurs by an unknown process that is thought to be electrochemically unfavourable (Swanson & Solomon, 1973).

In the present paper the plasmalemmal orientations of Mg/Ca-ATPase, (Na + K)-ATPase and adenylate cyclase activities have been examined by using enzymically dispersed pancreatic cells and the slowly permeant covalent probe diazotized sulphanilic acid. In contrast with (Na + K)-ATPase and adenylate cyclase activites, 90–95% of the plasma-membrane Mg/Ca-ATPase activity is expressed as an ecto-enzyme. These observations suggest that Mg/Ca-ATPase is probably not directly involved in ion transport in rat pancreas.

Abbreviations used: Mg/Ca-ATPase, (Mg^{2+} or Ca^{2+})-activated adenosine triphosphatase; (Na + K)-ATPase, $(Na^+ + K^+)$-activated ATPase; $(Ap)_5A$, [adenylyl-(3 → 5)]$_5$-adenosine; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; DSA, diazotized sulphanilic acid.

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Experimental

Materials

Concanavalin A, lentil lectin, collagenase, hyaluronidase, trypsin, pyruvate kinase, ouabain and all nucleotides and phosphate substrates were

from Sigma Chemical Co. $(Ap)_5A$ was from Boehringer Mannheim. Sulphanilic acid and sodium vanadate were from Fisher Scientific Co. Ancillary reagents were ACS grade or better. Pure secretin was generously given by Professor V. Mutt, Karolinska Institute, Stockholm, Sweden.

Preparation and isolation of plasma membranes

This was performed by modification of the method previously described (Martin & Senior, 1980). Briefly, defatted pancreata from 24 350g male rats were homogenized at 4°C with a Polytron homogenizer and microsomes collected by differential centrifugation. The microsomal material was resuspended in buffered sucrose and layered under discontinuous sucrose density gradients formed by successive layers of buffered 10, 27, 34 and 40% (w/v) sucrose at pH 7.5. The microsomes were centrifuged to equilibrium and the membranes collecting at the 10/27, 27/34, 34/40% interfaces and in the 40% pellet were collected and washed in buffered media containing 0.25 M-sucrose. Membranes were stored on ice and used within 24h of preparation. The term 'plasma membranes' subsequently used in the present paper refers to membranes collected at the 27/34% sucrose-density-gradient interface. This material exhibits the highest enrichment of Mg/Ca-ATPase relative to the whole homogenate and was enriched in (Na + K)-ATPase and secretin-sensitive adenylate cyclase activities.

Preparation of dispersed pancreatic cells

Enzymic dispersion of defatted pancreata was performed exactly as described by Amsterdam & Jamieson (1974). Cell viability was 96% on the basis of Trypan Blue exclusion. The morphological characteristics of the isolated cells were as described by Amsterdam & Jamieson (1974). The term 'intact cells' is subsequently used to identify this preparation.

Enzyme assays

Mg/Ca-ATPase. Activity in isolated membrane fractions was determined at 37°C in a 1 ml reaction mixture containing (final concentrations) 5 mM-$MgCl_2$ (or $CaCl_2$ as appropriate), 5 mM-Na_2ATP, 0.1 mg of bovine serum albumin and 75 mM-Tes/NaOH, pH 7.5. The reaction was initiated either by addition of 2–15 μg of membrane protein or of substrate as appropriate and terminated after 1 or 5 min (as indicated) by addition of 1 ml of 10% SDS. Liberated P_i was determined by the method of King (1932). Apparent K_m values for substrates were determined as previously described (Martin & Senior, 1980). Activity in isolated cells was determined by incubating 10^6 cells in 1.0 ml of a solution containing 120 mM-NaCl, 5 mM-KCl and 20 mM-Hepes/NaOH, pH 7.5, at 37°C. Reaction was initiated by addition of 5 mM-Mg- or Ca-ATP. Incubations (1–10 min as indicated) were terminated by the addition of SDS, and liberated P_i determined as described above.

(Na + K)-ATPase. Activity of isolated membrane fractions was determined at 37°C in 1.0 ml of 20 mM-KCl, 100 mM-NaCl, 5 mM-MgATP (vanadate-free), 75 mM-Tris/H_2SO_4, pH 7.5. The reaction also contained 100 μg of Zwittergent 3-12 (Calbiochem). This detergent selectively inactivates 95–97% of Mg/Ca-ATPase activity and enables a more accurate estimation of (Na + K)-ATPase, which is present at much lower initial specific activity. (Na + K)-ATPase activity was defined as the difference of activity in the presence and absence of 1 mM-ouabain.

Adenylate cyclase. Basal activity of isolated plasma-membrane fractions was determined by modification of the procedure described by Poirier *et al.* (1977). Aliquots (200 μl) of reaction mixture at 37°C, containing 10 mM-theophylline, 1 mM-Na_2GTP, 5 mM-$MgCl_2$, 10 mM-potassium phosphoenolpyruvate, 3 mM-Na_2ATP, 30 μg of pyruvate kinase/ml and 25 mM-Tris, pH 7.45, were mixed with 100–200 μg of membrane protein. Incubation for 10 min was terminated by boiling for 2 min. Blanks were boiled at zero time to correct for endogenous cyclic AMP. Reaction was linear for 10 min, but declined thereafter. Boiled samples were centrifuged at 8500 *g* for 2 min to pellet aggregated protein and the supernatant was removed for determination of cyclic AMP with a kit (Boehringer Mannheim). Secretin (28 nM) or NaF (10 mM) were added to the incubation mixture as required.

Lactate dehydrogenase. Activity was measured as described by Hohorst (1970) by continuously recording the increase in absorbance at 340 nm due to reduction of NAD upon addition of intact cells (~10^6 cells/assay), osmotically shocked cells, homogenates or isolated plasma-membrane fractions (100–300 μg/assay).

Other assays

Protein was measured by the procedure of Lowry *et al.* (1951), as modified by Miller (1959). Cell counts were performed by haemocytometry.

Preparation and use of diazotized sulphanilic acid (DSA)

DSA was prepared as described by DePierre & Karnovsky (1974*a*). Washed intact cells (~10^7 cells) were incubated at 37°C for 15 min in 1.0 ml of 120 mM-NaCl/5 mM-KCl/20 mM-Hepes/NaOH, pH 7.5, with and without 1 mM-DSA. Cells were subsequently washed twice in the above solution. Samples were removed for assay of Mg/Ca-ATPase and lactate dehydrogenase. In similar experiments, washed cells were osmotically shocked by resuspension in 20 mM-Hepes/NaOH, pH 7.5, and subse-

quently incubated in the presence or absence of 1 mM-DSA as described above.

Isolation of plasma membranes from dissociated cells

Freshly dissociated cells were incubated in Hepes-buffered saline ± 1 mM-DSA as described above. Cells (~7×10^9) were washed twice and subjected to Polytron homogenization as described for the preparation of plasma membranes above. The homogenates were assayed for Mg/Ca-ATPase and lactate dehydrogenase activities and subsequently fractionated by differential and sucrose-density-gradient centrifugation as described above.

Results

Features of ATP hydrolysis by isolated cells and plasma membranes

Fig. 1 shows the time course of hydrolysis of MgATP and MgADP in intact cells and plasma membranes. The time course of ATP hydrolysis was non-linear for both intact cells and plasma-membrane preparations, and decreased over 5 min to a rate of one-half that obtained over the first minute. Inclusion of an ATP-regenerating system consisting of phosphoenolpyruvate and pyruvate kinase did not alter the time course, suggesting that product inhibition by ADP was not responsible for the decrease in activity. The initial rate of ATP hydrolysis in the presence of Mg^{2+} was approx. 60–65 μmol of P_i/h per 10^7 cells for intact cells and up to 30 μmol of P_i/min per mg of protein for plasma-membrane fractions. Rates of MgADP hydrolysis were typically 20–25% of that observed with MgATP. The adenylate kinase inhibitor $(Ap)_5A$ at 1 mM did not affect the rate of MgADP hydrolysis by intact cells or plasma membranes. Rates of ATP and ADP hydrolysis essentially similar to those described above were obtained if Ca^{2+} replaced Mg^{2+} in the assays. ATP and ADP were hydrolysed at insignificant rates in the absence of bivalent cations. Other nucleotides, such as GTP, UTP, ITP and CTP, were hydrolysed (in the presence of optimal Mg^{2+} or Ca^{2+}) at rates 110, 92, 94 and 97% respectively of that observed with MgATP using intact cells or plasma membranes. Glucose 6-phosphate, *p*-nitrophenyl phosphate, AMP and PP_i or tripolyphosphate were hydrolysed at rates less than 1% of that of MgATP. These results indicated that the Mg/Ca-ATPase is a specific nucleoside di- or tri-phosphatase with broad specificity. Furthermore, the initial reaction products from MgATP as substrate were ADP and P_i as shown by t.l.c., and reaction products from MgADP were AMP and P_i. No evidence was found for PP_i liberation from MgATP or MgADP when thin-layer techniques previously described (Martin & Senior, 1980) were used. These data suggest, therefore, that the Mg/Ca-ATPase is not a nucleoside pyrophosphatase.

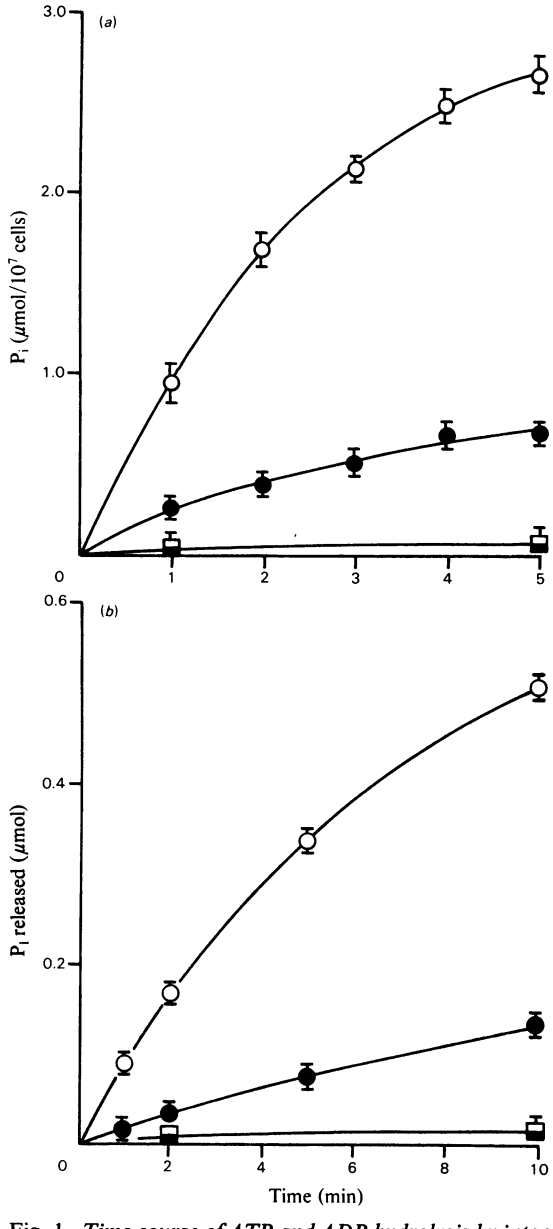

Fig. 1. *Time course of ATP and ADP hydrolysis by intact cells and plasma membranes*

The assay mixture contained (*a*) in 1.0 ml, 10^6 cells, or (*b*) in 2.0 ml, 2.7 μg of plasma membranes. Additions: ○, 5 mM-MgATP; ●, 5 mM-MgADP; □, 5 mM-ATP; ■, 5 mM-ADP. Each point represents the mean ± S.E.M. for two separate experiments, each in triplicate.

Activation of ATP hydrolysis by bivalent cations at fixed ATP concentration is shown in Fig. 2. Mg^{2+}

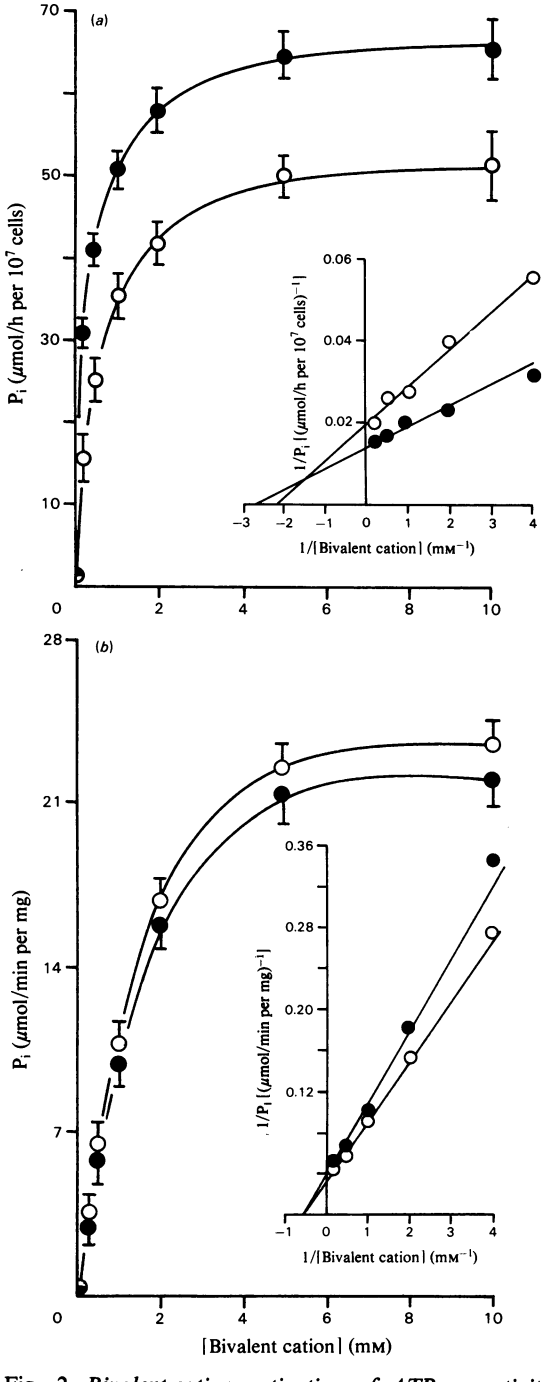

Fig. 2. *Bivalent-cation activation of ATPase activity associated with intact cells and plasma membranes*

The assay mixture contained 5 mM-ATP (fixed concentration) and (*a*) 10^6 cells for (*b*) 15 μg of plasma membranes. The reaction time was 1 min. Additions: ●, $MgCl_2$; ○, $CaCl_2$; each point represents the mean ± S.E.M. for three separate experiments, each in triplicate. The inset shows a Lineweaver–Burk plot of the data. (*a*) Cells: apparent K_m values: Mg, 350 μM; Ca, 450 μM; $V_{max.}$ values: Mg, 71.4; Ca, 56 μmol of P_i/h per 10^7 cells. (*b*) Plasma membranes: K_m values: Mg, 1.5 mM; Ca, 1.5 mM; $V_{max.}$ values: Mg, 25; Ca, 28 μmol of P_i/min per mg.

was somewhat more effective than Ca^{2+} at promoting ATP hydrolysis by intact cells, but not by plasma membranes. The effects of Ca^{2+} and Mg^{2+} were not additive at optimal concentrations, suggesting an action at the same site. Other ions, such as Na^+, K^+, Cl^-, HCO_3^- and SO_4^{2-}, were without effect on the ATPase activity of plasma membranes (Martin & Senior, 1980) and intact cells (results not shown). The activation of ATPase by bivalent-ion–ATP complexes in intact cells and plasma membranes is shown in Fig. 3. Both MgATP and CaATP were effective as substrates. The optimal concentration of substrates was similar (5 mM) for both preparations. Apparent K_m values were as follows: for cells, MgATP 310 μM; CaATP 290 μM; for plasma membranes, MgATP 310 μM; CaATP 260 μM. Consistent with the data in Fig. 2, addition of MgATP at optimal CaATP did not increase activity. No evidence was found for inhibition at high substrate concentrations. Intact cells and plasma membranes also hydrolysed MnATP and ZnATP at rates 30 and 4% respectively of that obtained with MgATP as substrate. The results showed that the Mg/Ca-ATPase activity of intact cells and isolated plasma membranes had essentially similar kinetic properties.

Action of inhibitors

The effects of well-characterized inhibitors of other ATPases were examined on the Mg/Ca-ATPase of intact cells and isolated plasma membranes. The results are presented in Table 1. The most striking feature is the similarity of response of Mg/Ca-ATPase to all the inhibitors in both preparations. The most effective inhibitors were mercuric chloride, *p*-chlormercuribenzoate and high concentrations of dicyclohexylcarbodi-imide and fluoride. The insensitivity of Mg/Ca-ATPase to inhibitors of ion-transporting ATPase such as (Na + K)-ATPase (ouabain, vanadate, *N*-ethylmaleimide), (H + K)-ATPase (F^-, *N*-ethylmaleimide), Ca-ATPase (*N*-ethylmaleimide, vanadate), and mitochondrial H^+-translocating ATPase (N_3^-, DCCD), demonstrates that the Mg/Ca-ATPase activity of intact cells and plasma membranes is different from the known ion-transporting ATPases. Of the four inhibitors of pancreatic HCO_3^- secretion tested (ouabain, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid, acetazolamide and amiloride), none was an effective inhibitor of Mg/Ca-ATPase. High concentrations of lead salts have been employed in histochemical techniques for localizing ATPase

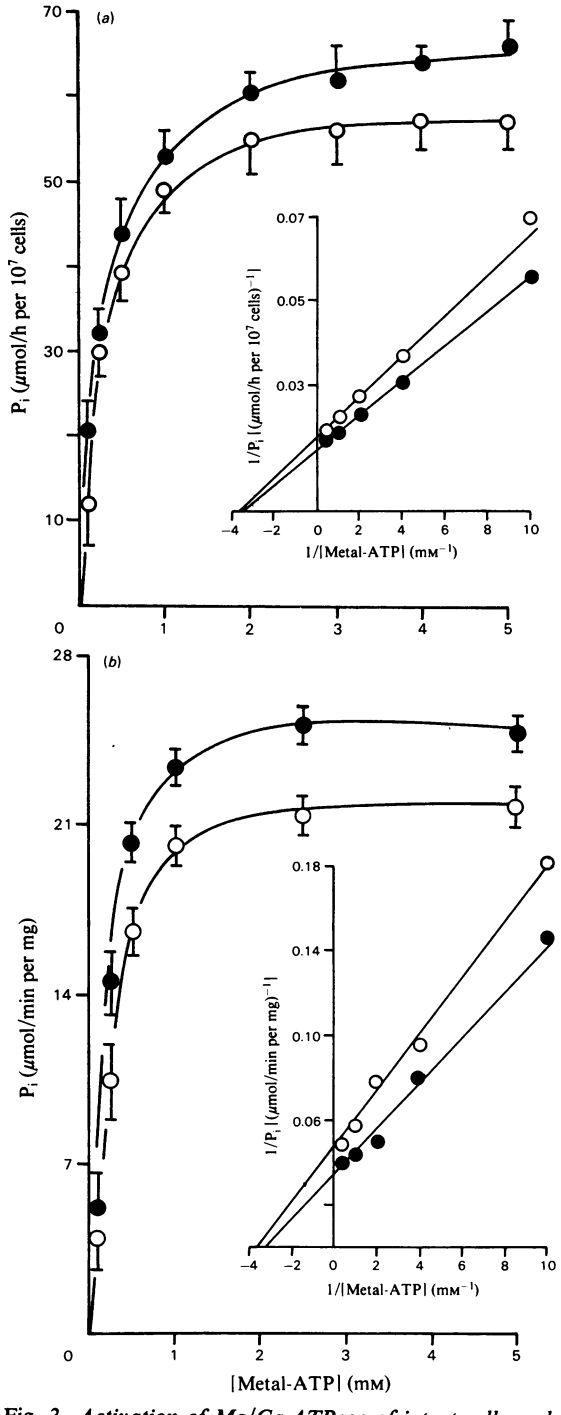

Fig. 3. *Activation of Mg/Ca-ATPase of intact cells and plasma membranes by MgATP and CaATP*

The assay mixture contained in 1 ml (*a*) 10^6 cells or (*b*) 8 μg of plasma membranes. The reaction time was 5 min. Additions: ●, MgATP; ○, CaATP. Each point represents the mean ± S.E.M. for three separate experiments, each in triplicate. The inset shows double-reciprocal plots of the data. (*a*) Cells: apparent K_m: MgATP, 285 μM; CaATP, 250 μM; $V_{max.}$: MgATP, 71; CaATP, 59 μmol of P_i/h per 10^7 cells. (*b*) Plasma membranes: apparent K_m: MgATP, 310 μM; CaATP, 260 μM; $V_{max.}$: MgATP, 28; CaATP, 21 μmol of P_i/min per mg.

activities *in situ* in the pancreas (Koenig *et al.*, 1976). Similar concentrations used in the present study were ineffective at inhibiting Mg/Ca-ATPase activity of intact cells and plasma membranes.

Inactivation of Mg/Ca-ATPase with DSA

Incubation of intact cells or plasma membranes with DSA for 15 min at 37°C irreversibly destroyed Mg/Ca-ATPase activity, as shown in Fig. 4. The Mg/Ca-ATPase activity of intact cells and plasma membranes were substantially inactivated by concentrations of DSA at 1 mM and above. The effect of DSA was irreversible in that extensive washing of cells and plasma membranes failed to restore any of the lost activity. Treatment of intact cells with DSA inactivated both Mg^{2+}- and Ca^{2+}-activated ATPase activities, as demonstrated in Table 2. The lactate dehydrogenase activity of intact cells was negligible. When cells were homogenized before DSA treatment, substantial lactate dehydrogenase activity was apparent, which was in turn destroyed by subsequent DSA treatment. DSA at 1 or 5 mM decreased

Table 1. *Effect of inhibitors on Mg/Ca-ATPase activity of intact cells and plasma membranes*

All assays were performed with 5 mM-MgATP. Similar results were obtained with CaATP as substrate. Plasma membranes were isolated from intact tissue as starting source. Abbreviations used: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid; NEM, *N*-ethylmaleimide; DCCD, dicyclohexylcarbodi-imide; *p*-CMB, *p*-chlormercuribenzoate.

Inhibitor	Concn. (mM)	ATPase activity remaining (%) Intact cells	Plasma membranes
Ouabain	1.0	96	98
Vanadate	0.1	97	96
SITS	0.1	90	87
	0.01	99	96
N_3^-	1.0	87	91
F^-	1.0	89	92
	10.0	59	50
$Pb(NO_3)_2$	10.0	99	97
	25.0	90	92
Acetazolamide	5.0	98	98
Amiloride	1.0	94	92
NEM	5.0	94	98
DCCD	0.5	18	10
$HgCl_2$	0.07	67	50
p-CMB	0.1	69	50

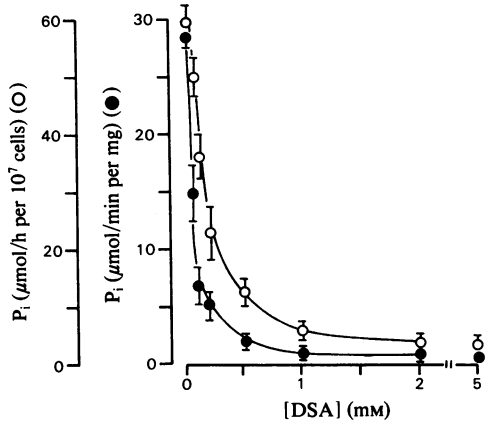

Fig. 4. *Inactivation of Mg/Ca-ATPase activity of plasma membranes and intact cells by DSA*

(●) Washed plasma membranes (~10 μg/assay) were resuspended in 120 mM-NaCl/5 mM-KCl/20 mM-Hepes/NaOH, pH 7.5, containing 1 mM-ouabain. DSA was added to the membranes at the indicated concentrations and the mixture incubated for 15 min at 37°C. Reaction mixtures were cooled on ice and portions were removed for Mg/Ca-ATPase assay as described in the Experimental section, with MgATP as substrate. (○) Washed intact cells (~10^6/assay) were incubated in Hepes-buffered saline (see the text) with DSA as described for plasma membranes above. At completion of incubation, cells were washed twice with buffered saline and the Mg/Ca-ATPase activity was determined. Results are expressed as μmol of P_i/h per 10^7 cells. In both cases the results are means ± S.E.M. for two experiments, each in triplicate.

Table 2. *Effect of DSA on ATPase and lactate dehydrogenase activities of intact cells and homogenates*

Each value is the mean ± S.E.M. for three separate experiments each performed in triplicate.

	Activity (μmol/h per 10^7 cells)		
	ATPase		Lactate dehydrogenase
Treatment	+ Mg	+ Ca	
Intact cells			
Untreated	60 ± 4	73 ± 3	< 0.1
+ 1 mM-DSA	7 ± 2	5 ± 1	< 0.1
+ 5 mM-DSA	3.5 ± 1	3.5 ± 2	< 0.1
Cells homogenized before DSA treatment			
Untreated	68 ± 7	82 ± 5	18.5 ± 3
+ 1 mM-DSA	9 ± 3	12 ± 2	2 ± 1
+ 5 mM-DSA	3 ± 2	2 ± 2	< 0.1
Cells homogenized after DSA treatment			
Untreated	67 ± 5	81 ± 3	17.6 ± 4
+ 1 mM-DSA	10 ± 3	12 ± 4	16.7 ± 4
+ 5 mM-DSA	3 ± 2	6 ± 2	14.3 ± 4

the ATPase activity of cells either pre-exposed or not pre-exposed to the probe to 20 and 13% respectively of their relevant control values. However, the intracellular marker, lactate dehydrogenase activity, was essentially unaffected by DSA treatment of cells before homogenization, but was greatly decreased (>90%) when exposed to the probe after homogenization. These results are similar to those of DePierre and Karnovsky (1974*a*,*b*) on ecto-phosphohydrolase activities and Carraway *et al.* (1980) on ecto-ATPase activities in mammary-gland tumour cells. Our results therefore support the hypothesis of an externally orientated active site for pancreatic Mg/Ca-ATPase. However, although DSA is relatively impermeant, it seems possible that sufficient quantities of the probe might cross the plasmalemma and reach sufficiently high localized concentrations at the intracellular face of the plasma membrane to react with membrane-bound enzymes whose active sites face the cytoplasm. In this situation, bulk intracellular marker-enzyme activity, as typified here by lactate dehydrogenase, would survive. Consequently, the failure of DSA to inactivate the intracellular lactate dehydrogenase activity of intact cells exposed to the probe before homogenization might lead to a false conclusion regarding the localization of Mg/Ca-ATPase activity. In order to clarify this potential problem, the activities of (Na + K)-ATPase and adenylate cyclase in isolated plasma-membrane fractions were determined by using material obtained from untreated or DSA-treated intact cells. Since the active sites of both these membrane-bound enzymes are known to face the cytoplasm, treatment of intact cells with DSA should have an insignificant effect on (Na + K)-ATPase and adenylate cyclase activities of subsequently isolated plasma membranes unless substantial amounts of DSA were permeating to the intracellular face of the plasma membrane during incubation of intact cells with the probe. The results of this study are shown in Table 3. For comparison, assays of plasma membranes isolated under three different conditions are given. Firstly, the isolation of plasma-membrane fractions from intact tissue as described in the Experimental section yields a Mg/Ca-ATPase of high specific activity (7–30 μmol of P_i/min per mg) at the 27/34 and 34/40% sucrose-density-gradient interfaces. (Na + K)-ATPase and adenylate cyclase activity were enriched in these fractions. The specific activities of Mg/Ca-ATPase in plasma-membrane fractions isolated from untreated intact cells was lower than that obtained with intact tissue as starting source and most likely represent inactivation due to the enzymic digestion of intact tissue required to produce a

Table 3. *Effect of DSA on ATPase and adenylate cyclase activities of isolated membrane fractions*
Density-gradient fractions refer to material banding at the interface formed by the indicated percentage-sucrose solutions. Adenylate cyclase was assayed as described in the Experimental section as basal activity, activity in the presence of 10mM-NaF, and activity in the presence of 28nM-secretin. Distribution of total protein among the membrane fractions isolated from intact cell homogenates were similar (within 5–10% for each respective fraction) for both untreated and DSA-treated intact cells. More than 95% of the enzymic activities measured in the plasma-membrane fractions were inactivated by exposure to 1mM-DSA in subsequent experiments. Values are means ± S.E.M. for three experiments, each in triplicate.

	ATPase activity (μmol/min per mg)		Adenylate cyclase activity (pmol/min per mg)		
Protocol	Mg/Ca	Na + K	Basal	+ 10mM-NaF	+ Secretin
Plasma membranes from intact tissue					
Untreated					
Whole homogenate	0.18 ± 0.03	0.005 ± 0.001	2.8 ± 0.2	18.9 ± 1.6	19.6 ± 2.3
Microsomes	1.4 ± 0.1	0.05 ± 0.01	13 ± 3	74 ± 9	68 ± 8
Density-gradient fraction					
27/34%	30 ± 4*	0.07 ± 0.02	75 ± 8	405 ± 50	480 ± 62
34/40%	7 ± 2	0.25 ± 0.06†	16 ± 3	106 ± 13	170 ± 20
Plasma membranes from intact cells					
(*a*) Untreated					
Microsomes	2.2 ± 0.6	0.050 ± 0.01	10 ± 2.4	54 ± 9	53 ± 7
27/34%	7.0 ± 5	0.24 ± 0.07	50 ± 8	291 ± 27	288 ± 31
34/40%	6.0 ± 2	0.15 ± 0.04	9 ± 1.2	63 ± 7	90 ± 14
(*b*) + 1mM-DSA pretreated					
Microsomes	0.24 ± 0.1	0.048 ± 0.01	9.1 ± 2.6	50 ± 11	49 ± 7
27/34%	0.73 ± 0.4	0.23 ± 0.08	42 ± 5	241 ± 27	268 ± 32
34/40%	0.62 ± 0.15	0.12 ± 0.03	8.1 ± 1.6	58 ± 6	7 ± 11

* Typical enrichment of this activity was 160 relative to whole homogenate.
† Typical enrichment was 50 relative to whole homogenate.

dispersed cell preparation. The specific activities of (Na + K)-ATPase and adenylate cyclase, however, were comparable with those obtained by using intact tissue as starting material. Isolation of plasma membranes from intact cells treated with 1mM-DSA showed that the specific activities of (Na + K)-ATPase and adenylate cyclase were 80–90% of those observed in membranes isolated from untreated intact cells. Moreover, the responsiveness of adenylate cyclase to added secretin indicates that the receptor–cyclase coupling had survived the prior DSA treatment. However, 90–95% of the specific activity of Mg/Ca-ATPase was inactivated by the prior DSA treatment of intact cells. This evidence provides additional support for the hypothesis that the Mg/Ca-ATPase of intact rat pancreatic cells is an ecto-enzyme. By using the ratio of specific activities of plasma-membrane fractions derived from DSA-treated cells versus untreated cells, it is possible to obtain an estimate of the activity expressed as an ecto-enzyme, as shown in Table 4. On the basis of this calculation, 90–95% of the Mg/Ca-ATPase activity that is collected at the 10/27, 27/34 and 34/40% sucrose-density interfaces is referable to as an ecto-enzyme. In these same fractions, less than 20% of (Na + K)-ATPase and adenylate cyclase activities were manifest as ecto-enzymes, and this value is probably an overestimate, owing to likely inactivation by small quantities of DSA that may have penetrated the plasma membrane.

Effects of concanavalin A on the Mg/Ca-ATPase activity of intact cells and plasma membranes

The external surface of many plasma-membrane proteins may be extensively glycosylated, and many studies have explored the effects of plant lectins such as concanavalin A on the activity of a number of ecto-enzymes (Carraway *et al.*, 1976; 1980). Concanavalin A was effective in stimulating the Mg/Ca-ATPase activity associated with both intact cells and plasma-membrane fractions (Table 5). The concanavalin A effect was concentration-dependent and exhibited positive co-operativity ($h = 1.8$). The stimulatory effect of the lectin was blocked by the antagonist α-methyl D-mannoside. Concanavalin A stimulates ATPase activity in both preparations by linearizing the relationship between ATP hydrolysis and time (cf. Fig. 1). No significant effects of concanavalin A were found on the initial rate (0–1 min) of ATP hydrolysis. Furthermore, the degree of non-linearity of ATP hydrolysed versus time in Fig. 1 was found to vary as a function of the assay temperature. At assay temperatures below

Table 4. *Calculated extracellular distribution of enzymic activities associated with plasma membranes from intact cells*
The outside activity was calculated as:

$$100 - \left(\frac{\text{Specific activity of plasma membranes from DSA-treated cells}}{\text{Specific activity of plasma membranes from untreated cells}} \times 100 \right)$$

The derived orientations are based on that activity accessible or inaccessible to DSA. The distribution of total protein in the derived plasma-membrane fractions was similar for DSA-treated or untreated cells.

	Outside activity (%)		
	ATPase		
	Mg/Ca	Na + K	Adenylate cyclase
Microsomes	90	4	9
Density-gradient fraction			
27/34%	91	4	15
34/40%	91	20	10

Table 5. *Effect of concanavalin A on Mg/Ca-ATPase of intact cells and plasma membranes of pancreas*
Intact cells (10^6/assay) or plasma membranes (6 μg) were preincubated for 1 min at 37°C with concanavalin A before initiation of reaction with 5 mM-MgATP. The reaction time was 5 min. Each value is the mean of four determinations on one preparation each. Abbreviation used: α-MM, α-methyl D-mannoside. Similar results were obtained with CaATP as substrate.

[Concanavalin A] (μg/ml)	ATPase activity (%)	
	Intact cells	Plasma membranes
0	100	100
0.2	105	112
0.5	113	125
1.0	121	136
5.0	160	188
50.0	178	197
100.0	197	205
500.0	224	218
500.0 + 25 mM-α-MM	109	119
0 + 25 mM-α-MM	102	103

30°C the ATPase reaction was linear with time and concanavalin A had no effect on ATPase activity. Interestingly, at all assay temperatures studied (23–45°C), the ADPase activity associated with cells and membranes was unaffected by concanavalin A. In other experiments, the lectin from *Lens culinaris* gave similar results. We have not explored the nature of the sugars involved in this effect. Carraway *et al.* (1980) have reported that concanavalin A induced an apparent stimulation of the ecto-ATPase activity associated with mammary-adenocarcinoma-cell plasma membranes by relieving substrate inhibition by ATP. We found no evidence for this effect in our preparations. In other experiments using plasma membranes at 37°C and 5 min assay times, concanavalin A at 5 μg/ml increased the apparent K_m for MgATP from 300 μM to 420 μM and increased $V_{max.}$ from 30 to 45 μmol/min per mg.

Discussion

On the basis of the criteria described by DePierre & Karnovsky (1974*a*) the pancreatic Mg/Ca-ATPase appears to be an ecto-enzyme. An external localization of the active site of this enzyme is supported by the following lines of evidence.

(1) Externally added ATP was hydrolysed at high rates by cell populations containing approx. 4–5% non-viable cells (as determined by Trypan Blue exclusion). Energy-dependent ATP uptake was excluded, since treatment of intact cells with protonophores (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), azide or oligomycin as mitochondrial poisons does not affect the rate of extracellular ATP hydrolysis.

(2) Assay of the media in which cells were previously suspended showed no significant hydrolysis of added ATP, suggesting that ATP hydrolysis was not due to a secreted enzyme.

(3) The total Mg/Ca-ATPase activity determined in the intact cell represented a high proportion of that obtainable in the whole homogenate.

(4) Treatment of intact cells for short intervals with the slowly penetrating covalent reagent DSA destroyed the activity of the putative ecto-enzyme, but left intact the activities of intracellular cytoplasmic and plasma-membrane markers such as lactate dehydrogenase, (Na + K)-ATPase and adenylate cyclase. Similarly, subsequent treatment of cell homogenates with DSA under similar conditions led to inactivation of the intracellular marker activities mentioned above.

(5) The plasma-membrane fraction obtained from dispersed cells showed a distribution and activity of

plasma-membrane markers similar to that obtained with whole tissue as a starting source, and showed that the prior treatment of intact dispersed cells, either with or without DSA, resulted in extensive loss of the ecto-enzyme activity (Mg/Ca-ATPase), but not of the other activities in subsequently isolated plasma-membrane fractions.

(6) The enzyme activities of plasma-membrane fractions isolated from whole tissue or dispersed cells not previously treated with DSA were sensitive to subsequent treatment with DSA.

The pancreatic Mg/Ca-ATPase characterized in the present study is clearly different from the other major cation transporting ATPases. Its lack of sensitivity to ouabain, vanadate, azide, *N*-ethylmaleimide, Ruthenium Red and univalent cations distinguish this activity from (Na + K)-ATPase, Ca^{2+}-activated ATPase, gastric (H + K)-activated ATPase and mitochondrial H^+-translocating ATPase. In addition, the major cation-transporting enzymes have active sites facing the cytoplasm. Furthermore, the ATPase activated by Mg^{2+} or Ca^{2+} represents the major ATP-hydrolysing activity of intact rat pancreatic cells and plasma-membrane fractions. Both the Mg^{2+}- and Ca^{2+}-stimulated ATPase activity of plasma membranes co-enrich in the same fractions on sucrose density gradients and both activities show parallel behaviour towards concanavalin A and inhibitors. The simplest explanation consistent with the data is that the Mg-ATPase and Ca-ATPase activities of intact cells and plasma membranes reflect the action of a single ecto-enzyme. Similar ecto-ATPases have been reported with Erhlich-ascites-carcinoma cells (Wallach & Ullrey, 1962; Ronquist & Agren, 1975), intact human blood platelets (Chambers *et al.*, 1967), rat mammary-gland cells (Carraway *et al.*, 1980), guinea-pig polymorphonuclear leucocytes (DePierre & Karnovsky, 1974*a*,*b*), neuroblastoma and glioma cells (Stefanovic *et al.*, 1976), human granulocytes (Smolen & Weissmann, 1978) and other cell lines (Trams & Lauter, 1974). The present paper is the first to document an ecto-ATPase in the pancreas.

Previous studies have demonstrated the presence of an HCO_3^--stimulated Mg-ATPase activity in plasma membranes isolated from pancreas (Simon & Thomas, 1972; Simon *et al.*, 1972; Weichmann *et al.*, 1974), and have implicated this activity in the pancreatic secretion of HCO_3^-. However, subsequent work (Van Amelsvoort *et al.*, 1978; Martin & Senior, 1980), demonstrated that previous reports of an HCO_3^--activated ATPase in plasma-membrane fractions from pancreas were most likely due to contamination by mitochondrial membranes. By using improved techniques, it was shown that Mg/Ca-ATPase is present at high specific activity in plasma-membrane fractions from rat pancreas (Martin & Senior, 1980) and initially it appeared plausible that this enzyme might be involved in the secretion of pancreatic HCO_3^- that is thought to be electrochemically unfavourable (Swanson & Solomon, 1973) and thus energy-requiring. However, the present results suggest that the Mg/Ca-ATPase of rat pancreas is expressed as an ecto-enzyme, and it is now difficult to conceive of a role for this activity in pancreatic HCO_3^- secretion. Moreover, as shown here, inhibitors of pancreatic HCO_3^- secretion *in vitro* or *in vivo*, such as Na^+ or K^+ omission, amiloride, ouabain, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid and acetazolamide (for review, see Case *et al.*, 1980) had no effect on the activity of Mg/Ca-ATPase from intact cells or plasma membranes (Table 1).

The physiological function(s) of ecto-ATPase activity is not clear, but may simply serve to regulate extracellular ATP concentration in the vicinity of the plasma membrane. Possible functions of ecto-enzymes and ATPases have been extensively discussed by DePierre & Karnovsky (1974*b*). With transformed cells (Trams, 1974; Rozengurt & Heppel, 1975) and isolated kidney tubules (Rorive & Kleinzeller, 1972), the addition of nucleoside triphosphates increased membrane permeability to ions. Trams (1974) has suggested that cells may release ATP during stimulation and that an ecto-ATPase might mediate membrane properties directly or by terminating the ATP signal by hydrolysis. More recent evidence has shown that external ATP stimulates amylase release from superfused mouse parotid glands in the presence of atropine, phentolamine and propranolol, an effect blocked by quinidine (Gallacher, 1982). Unless ATP were released from nerve terminals or by an unknown cellular-secretion mechanism, it is difficult to see, *in vivo*, how sufficient concentrations could accumulate for it to act as a local secretagogue. Furthermore, plasma levels of ATP have been estimated at 1 μM or less (Brashear *et al.*, 1968; Forrester, 1969).

Finally, no role for extracellular nucleotide has been demonstrated in the pancreas, and the function of the ecto-Mg/Ca-ATPase activity that we have described remains to be clarified.

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