Nucleotide and bivalent cation specificity of the insulin-granule proton translocase

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1. The nucleotide and bivalent cation specificity of the proton translocase activity of insulin secretory granules was investigated by assessing the inhibitor-sensitive rates of nucleotide hydrolysis by these organelles in relation to their chemiosmotic properties. 2. The relative rates of nucleotide hydrolysis by freeze/thawed granule preparations were: Mg^{2+}ATP (100%) > Mg^{2+}GTP (55%) > Mg^{2+}UTP (48%) > Mg^{2+}ITP (44%) > Mg^{2+}CTP (23%) > Mg^{2+}TTP (20%), and by intact granules were: Mg^{2+}ATP (100%) > Mg^{2+}ITP (74%) > Mg^{2+}GTP (60%) > Mg^{2+}CTP (35%). Mg^{2+}ATP , Mg^{2+}GTP and Mg^{2+}ITP hydrolyses were inhibited by tributyltin and stimulated, in intact granules, by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mg^{2+}CTP hydrolysis was not markedly affected by these compounds. Correspondingly, only Mg^{2+}ATP , Mg^{2+}GTP and Mg^{2+}ITP produced large changes in the $\Delta\psi$ and $\Delta\mu_{\text{H}^+}$ across the granule membrane. 3. The relative rates of maximal ATPase activity stimulated by bivalent cations in freeze/thawed granule preparations were: Mg^{2+} (100%) > Mn^{2+} (82%) > Ca^{2+} (40%) > Co^{2+} (36%) > Zn^{2+} (0%), and in intact granules were: Mg^{2+} (100%) > Mn^{2+} (85%) > Co^{2+} (61%) > Ca^{2+} (42%). Tributyltin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone affected Mg^{2+} -, Mn^{2+} - and Co^{2+} -activated, but not Ca^{2+} -activated, ATP hydrolysis. Correspondingly, only Mg^{2+} , Mn^{2+} and Co^{2+} supported the generation of a $\Delta\psi$ and $\Delta\mu_{\text{H}^+}$ across granule membranes in the presence of ATP. 4. The results were consistent with a single proton translocase that had its catalytic site exposed on the external face of the granule membrane. The indicated specificity ($\text{Mg}^{2+}\text{ATP} = \text{Mn}^{2+}\text{ATP} > \text{Co}^{2+}\text{ATP} > \text{Mg}^{2+}\text{GTP} > \text{Mg}^{2+}\text{ITP}$) was similar to that of enzymes described in membrane fractions prepared from adeno-hypophyseal tissue, adrenal chromaffin granules and yeast vacuoles. The insulin-granule activity thus appears to be a type of proton translocase, which is characteristic of intracellular storage vesicles in eukaryotic cells.

A proton-translocating Mg^{2+} -dependent ATPase is recognized as an important component in the process of biogenic amine accumulation in the storage granules of adrenal medullary cells (see Njus *et al.*, 1981) and platelets (Johnson *et al.*, 1978). More recently, a similar enzymic activity has been detected in storage granules from tissues that secrete polypeptide hormones, notably in neurohypo-

physeal tissue (Russell & Holz, 1981) and in B-cells of pancreatic origin (Hutton & Peshavaria, 1982).

In both adrenal chromaffin and insulin storage granules inwardly-directed transport of H^+ results either in the formation of a $\Delta\psi$ across the granule membrane positive with respect to the granule exterior or, if a permeant anion is present, the acidification of the granule interior. The rates of ATP hydrolysis by these granules are similar in spite of a marked difference in their capacities to store biogenic amines. It has been observed for chromaffin granule ghosts that the Mg^{2+} -dependent ATPase activity far exceeds the energetic requirements for amine transport (Phillips, 1974). This raises the

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Abbreviations used: ΔpH , transmembrane pH gradient; $\Delta\psi$, membrane potential; $\Delta\mu_{\text{H}^+}$, protonmotive force.

possibility that the major component of the ATPase activity serves some other function, one possibly related to granule biogenesis or exocytosis.

The present paper extends previous studies of the properties of the proton translocase of insulin secretory granules isolated from a transplantable rat insulinoma. The nucleotide and bivalent cation specificity of nucleotide hydrolysis are assessed in relation to the corresponding specificity for the formation of the ΔpH and $\Delta\psi$ across the granule membrane.

Experimental

Materials

[^{14}C]Methylamine hydrochloride (sp. radioactivity 60 Ci/mol), potassium thio[^{14}C]cyanate (sp. radioactivity 60 Ci/mol), inulin[^{14}C]carboxylic acid (sp. radioactivity 6 Ci/mol), $^3\text{H}_2\text{O}$ and carrier-free [^{32}P]P_i were obtained from Amersham International, Amersham, Bucks., U.K. [γ - ^{32}P]ATP was prepared enzymically by the method of Walsh *et al.* (1971) and purified by absorption on to 5 mg of activated charcoal, which was then washed with 2 ml of water and eluted with 100 μl of a solution of aq. NH_3 (0.15 M) in 50% (v/v) ethanol. Tri-*n*-butyltin chloride (tributyltin) was obtained from Ralph N. Emanuel, Alpert, Middx., U.K., and other biochemical reagents from Sigma (London) U.K., Kingston upon Thames, Surrey, U.K.

All stock solutions used to prepare homogenization media, density-gradient media and assay buffers were passed through columns (5 cm $\times$ 1 cm) of bivalent cation-chelating resin (Chelex; Bio-Rad, Richmond, CA, U.S.A.) before use. Plasticware was used throughout to avoid bivalent cation contamination.

Secretory granule preparation

Insulin secretory granules were prepared from a transplantable rat islet cell tumour (Chick *et al.*, 1977) by density-gradient centrifugation in Percoll-containing media (Pharmacia, Uppsala, Sweden) as previously described (Hutton & Peshavaria, 1982; Hutton *et al.*, 1982). The final preparation was suspended at 10 mg of protein/ml in a solution containing 0.25 M-sucrose and 10 mM-4-morpholine-ethanesulphonic acid that had been adjusted to pH 6.5 with Tris base. Granules were either maintained at 4°C for up to 4 h or stored at -70°C before use.

Freeze/thawed granule preparations were diluted to 1 mg of protein/ml in 10 mM-4-morpholine-ethanesulphonate buffer (pH 6.5) and passed through columns (0.5 cm $\times$ 1 cm) of Chelex (see above) to remove endogenous bivalent cations. Such treatment neither affected the recovery of protein nor enzymic activities from the samples. Atomic-absorption

spectroscopic determinations on four different preparations showed that the granule Ca^{2+} content was reduced to <8 nmol/mg of protein (<2 μM in the assay) and Mg^{2+} to 12 ± 3 nmol/mg (approx. 10 μM in the assay) from initial values in the range of 100–200 nmol/mg.

Enzymic assays

Nucleoside triphosphate hydrolysis by freshly prepared granules was assayed in a reaction mixture containing (final concentration) 0.25 M-sucrose, 50 mM-maleic acid/Tris buffer (pH 7), 2 mM-nucleotides and bivalent cations at concentrations of 2.5 mM or 1 mM (Co^{2+}). Incubations were initiated by the addition of 50 μl of granule suspension (0.5–1.0 mg of protein/ml) to 150 μl of reaction mixture contained in 3 ml-capacity polystyrene tubes (LP3 tubes; W. Sarstedt, Leics., U.K.) and terminated after 15 min at 37°C by adding 0.5 ml of ice-cold 7% (w/v) trichloroacetic acid. Samples were centrifuged at 4°C for 20 min and 1700 *g* and [P_i] in the supernatant fluid was determined spectrophotometrically (Fiske & SubbaRow, 1925). Assays on freeze/thawed granule preparations were performed by the same technique in a reaction mixture containing 50 mM-KCl and 50 mM-Tris/HCl (pH 7.5). Bivalent cations and nucleotides were used at the same final concentrations as above. However, the latter were added after an initial pre-incubation of the samples at 37°C for 15 min. Both assays were also adapted for the radiometric determination of ATPase activity by the method of Seals *et al.* (1978). In this instance, 20 μl of a reaction mixture containing radioactively labelled ATP (about 0.1 μCi) was mixed with 20 μl of sample in a 1.5 ml-capacity polypropylene tube (Eppendorf microcentrifuge tube; Sarstedt) and terminated after 15 min at 37°C by the addition of 10 μl of 5% (w/v) sodium dodecyl sulphate. Subsequent operations were as described in the original method except that reagent volumes were reduced to one-quarter of the previous volumes. The activities determined by this assay were identical with those recorded by the spectrophotometric procedure. Incubations containing all components except granule protein served as blank determinations in each assay. Correction for endogenous P_i (approx. 150 nmol/mg of protein) and P_i released from endogenous substrates (<5 nmol/mg) was also made in the case of spectrophotometric assays by performing incubations in the absence of added nucleotides; this resulted in an adjustment equivalent to <10% of the final absorbance recorded in the presence of Mg^{2+} -ATP. Tributyltin (2.5 μM), 25 μM -*NN'*-dicyclohexylcarbodi-imide and 5 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (final concentrations) were introduced into assay mixtures in 100-fold concentrated solutions in dimethyl sulph-

oxide; this solvent, however, did not affect the assays. Chelating agents, when used, were added as neutral K^+ salts. The concentrations of the free and complexed forms of nucleotides and bivalent cations were calculated by an iterative procedure (Storer & Cornish-Bowden, 1976) on an IBM 370/165 computer. Dissociation and metal-stability data obtained in the presence of 0.1 M-KCl were used in these calculations (Sillen & Martell, 1964).

Chemiosmotic parameters

The ΔpH and $\Delta\psi$ across the membrane of freshly-prepared granules were determined as described by Hutton (1982) from the spaces of distribution at equilibrium of [^{14}C]methylamine and thio[^{14}C]cyanate respectively. The intragranular concentration of these radioisotopes were calculated on the basis of the intragranular water space determined under identical incubation conditions from the difference in the spaces of distribution of 3H_2O and inulin[^{14}C]carboxylic acid. Incubation media contained 1–5 μCi of each radioisotope/ml, 0.25 M-sucrose, 25 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, 2 mM-nucleotides and bivalent cations at 2.5 mM or 1 mM (Co^{2+}) final concentrations. The microcentrifuge tubes (Eppendorf; Sarstedt) in which the incubations were conducted were pretreated with a solution of 2% (w/v) bovine serum albumin for 2 h and rinsed in water. The immiscible phase used to separate granules from their incubation medium was a mixture (1:1, v/v) of di-n-butyl phthalate and silicone fluid MS550 (BDH Chemicals, Poole, Dorset, U.K.). The methods were otherwise as originally detailed.

Protein was determined by the method of Lowry *et al.* (1951) after precipitation with 5% (w/v) trichloroacetic acid with bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard. The statistical significance of differences of means was estimated by Student's two-tailed *t* test for unpaired data.

Results

Nucleotide specificity

NTPase activity in freeze/thawed granules. Freeze/thawed granule preparations incubated in the presence of Mg^{2+} catalysed P_i formation from GTP, ITP, UTP, CTP and TTP at rates that were equivalent to 55, 48, 44, 23 and 20% respectively of that observed with ATP (Table 1). Tributyltin reduced P_i formation from ATP, GTP and ITP but was without effect on CTP hydrolysis. The concentration of the inhibitor used here was 10-fold that required for a half-maximal inhibitory effect on the Mg^{2+} -dependent ATPase activity (Hutton & Peshavaria, 1982).

Table 1. *Nucleotide hydrolysis by freeze/thawed granules and the effect of tributyltin*

Spectrophotometric assays were performed using a maleic acid/Tris buffer (pH 7) (see the Experimental section). Each tabulated value is the mean $\pm$ S.E.M. of results obtained with different granule preparations; the numbers of these are shown in parentheses. The statistical significance of differences refers to the effect of adding tributyltin (*, $P < 0.001$; **, $P < 0.05$). Abbreviation: N.D., not determined.

	P_i formation (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	
Mg^{2+} ATP	65.7 $\pm$ 5.5 (9)	19.7 $\pm$ 1.7 (4)*
Mg^{2+} GTP	36.2 $\pm$ 4.7 (6)	17.1 $\pm$ 0.8 (3)**
Mg^{2+} ITP	29.0 $\pm$ 3.1 (6)	17.4 $\pm$ 0.8 (3)**
Mg^{2+} CTP	15.0 $\pm$ 2.5 (6)	12.7 $\pm$ 0.9 (3)
Mg^{2+} UTP	31.8 $\pm$ 9.0 (3)	N.D.
Mg^{2+} TTP	13.4 $\pm$ 4.0 (3)	N.D.

Table 2. *Nucleotide hydrolysis by intact granules and the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide*

Rates of P_i formation catalysed by freshly-prepared granules were determined spectrophotometrically under iso-osmotic incubation conditions in maleic acid/Tris buffer (pH 7) (see the Experimental section). Each tabulated value is the mean $\pm$ S.E.M. of results obtained with four different granule preparations. The statistical significance of differences refer to the effect of adding carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide (*, $P < 0.001$; **, $P < 0.02$; ***, $P < 0.05$). Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide.

	P_i formation (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	
Addition	Control	Plus FCCP
Mg^{2+} ATP	50.9 $\pm$ 2.7	119.2 $\pm$ 6.8*
Mg^{2+} GTP	30.6 $\pm$ 2.9	50.7 $\pm$ 4.7**
Mg^{2+} ITP	37.6 $\pm$ 2.6	49.3 $\pm$ 3.5***
Mg^{2+} CTP	17.6 $\pm$ 0.7	21.8 $\pm$ 3.0

NTPase activity in intact granules. Freshly-prepared granules incubated under iso-osmotic conditions hydrolysed ITP, GTP and CTP in the presence of Mg^{2+} at rates equivalent to 74, 60 and 35% respectively of that observed with ATP (Table 2). The protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide enhanced the hydrolysis of ATP, GTP and ITP, but had little effect either on CTP hydrolysis or on P_i formation by granules incubated without exogenous nucleotides. The relative rates of hydrolysis of the different nucleotides in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide were similar to those expressed in freeze/thawed preparations (Table 1).

Table 3. *Nucleotide effects on the chemiosmotic properties of granules*

The uptake of [^{14}C]methylamine and thio[^{14}C]cyanate by freshly-prepared granules are expressed as their concentrations inside the granule relative to that of the medium. The ΔpH and $\Delta\psi$ were derived from these measurements, using the Nernst equation in the latter case; the $\Delta\mu_{\text{H}^+}$ is the sum of these components expressed in mV terms (Mitchell, 1961). Each tabulated value is the mean $\pm$ S.E.M. of results obtained from three to five different granule preparations; n represents the numbers of separate incubations performed. The statistical significance of differences refers in each case to incubations performed in the presence of Mg^{2+} alone (*, $P < 0.001$; **, $P < 0.05$).

Addition	n	Methylamine uptake ratio	Thiocyanate uptake ratio	ΔpH	$\Delta\psi$ (mV)	$\Delta\mu_{\text{H}^+}$ (mV)
Mg^{2+} alone	15	19.23 ± 1.76	1.40 ± 0.16	1.28	8.8	85.8
Mg^{2+}ATP	15	19.46 ± 1.41	$7.23 \pm 0.91^*$	1.29	51.5	128.9
Mg^{2+}GTP	11	17.76 ± 1.19	$6.31 \pm 0.78^*$	1.25	48.0	122.9
Mg^{2+}ITP	11	18.49 ± 1.16	$5.60 \pm 0.76^*$	1.27	44.8	120.9
Mg^{2+}CTP	11	$15.31 \pm 1.10^{**}$	$2.13 \pm 0.31^{**}$	1.19	19.7	90.8

Nucleotide effects on the chemiosmotic properties of the granule. None of the nucleotides tested affected appreciably the ΔpH across the granule membrane when comparison was made with the incubation condition with no nucleotides present (Table 3). All supported, however, the formation of a $\Delta\psi$, the most pronounced response being with ATP followed by GTP and ITP. Their effects on the $\Delta\mu_{\text{H}^+}$ were ranked similarly. The hierarchy of responses here paralleled that observed in the rates of P_i formation from different nucleotides in freeze/thawed granule preparations, the relative sensitivity of NTPase activity to tributyltin and the relative rates of nucleotide hydrolysis in intact granules in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Tables 1 and 2).

Bivalent cation specificity

Bivalent cation activation of ATP hydrolysis. Freeze/thawed granule preparations hydrolysed ATP in the absence of exogenous Mg^{2+} at a rate equivalent to $11.5 \pm 1.4\%$ (13 preparations) of that observed with $2.5\text{ mM-Mg}^{2+}\text{ATP}$. The addition of 0.5 mM-EGTA to the medium did not affect this basal rate. However, 0.5 mM-EDTA or $0.5\text{ mM-trans-1,2-diaminocyclohexanetetra-acetic acid}$ reduced it to $7.8 \pm 2.0\%$ ($n = 3$) and $3.7 \pm 1.2\%$ ($n = 7$) of the Mg^{2+} -dependent activity respectively. These effects were consistent with the presence of residual Mg^{2+} , but not Ca^{2+} , in the preparation (see the Experimental section).

Mg^{2+} , Mn^{2+} , Co^{2+} and Ca^{2+} in the sub-millimolar concentration range increased ATP hydrolysis by freeze/thawed preparations; above 2 mM , however, both Mn^{2+} and Co^{2+} were inhibitory (Fig. 1). Zn^{2+} did not support ATP hydrolysis at any concentration between $50\text{ }\mu\text{M}$ and 5 mM . At a 2 mM concentration, Zn^{2+} abolished ATP hydrolysis either in the absence of bivalent cations or in the presence of Mg^{2+} , Mn^{2+} , Co^{2+} or Ca^{2+} . The K_i determined in

Fig. 1. *Bivalent cation concentration dependency of ATPase activity in freeze/thawed granules*

Freeze-thawed granule preparations were assayed spectrophotometrically in Tris/HCl buffer (pH 7.5) (see the Experimental section). Results obtained with Mg^{2+} (●), Mn^{2+} (○), Co^{2+} (□), Ca^{2+} (■) and Zn^{2+} (▲) are shown.

the presence of 2.5 mM-Mg^{2+} and 2 mM-ATP was 0.3 mM .

Lineweaver-Burk plots of data obtained with Mg^{2+} under conditions identical with those described in Fig. 1 indicated a major activity with a K_a for Mg^{2+} of $150\text{ }\mu\text{M}$. When the Mg^{2+} concentration of the media was kept at 1 mM greater than the ATP concentration and the latter was varied, the K_m for

ATP was $145\text{ }\mu\text{M}$. The similarity of these values suggested that an Mg^{2+} -ATP complex was the actual enzyme substrate. Corresponding values for Ca^{2+} -activated ATP hydrolysis assayed in the same manner were 1.0 mM and 1.3 mM . This likewise suggested that a Ca^{2+} -ATP complex was being hydrolysed in this instance.

Ca^{2+} -activated ATP hydrolysis determined at $10\text{ }\mu\text{M}$ free ionized Ca^{2+} in the presence of EGTA by using the radiometric assay, or analysed at 0.5 mM free ionized Ca^{2+} under the conditions shown in Fig. 1, was not affected by the addition of $1.2\text{ }\mu\text{M}$ -bovine brain calmodulin to the assay or the inclusion of the calmodulin antagonist trifluoperazine ($50\text{ }\mu\text{M}$). Activity was also not affected by KCl in the concentration range of $1\text{--}50\text{ mM}$. In the presence of 2.5 mM-Mg^{2+} , no effect of Ca^{2+} ($10\text{ }\mu\text{M}$ or 0.5 mM) addition on the rate of ATP hydrolysis was observed by using either the radiometric or the spectrophotometric assay. Calmodulin again had no effect under these conditions (J. C. Hutton & M. Peshavaria, unpublished work). The Ca^{2+} -activated ATPase activity observed here thus displayed features characteristic of a high- K_m (Ca^{2+} or Mg^{2+})-activated enzyme distinct from the Ca^{2+} -activated Mg^{2+} -dependent ATPases involved in Ca^{2+} transport (see Schuurmans Stekhoven & Bonting, 1981).

ATPase activities expressed in the presence of Mg^{2+} , Mn^{2+} or Co^{2+} were inhibited by tributyltin and NN' -dicyclohexylcarbodi-imide (Table 4), in every instance being reduced to $<25\%$ of their initial rates. ATPase activity supported by Ca^{2+} , by contrast, was reduced only slightly, and only to an extent corresponding to the decrease that was evident in the basal rate.

Bivalent cation-activated ATPase activity in intact granules. The addition of Mg^{2+} , Mn^{2+} , Co^{2+} or Ca^{2+} to intact granules increased their ATPase activity by factors of 7.4, 6.3, 4.6 and 3.2 relative to the rate of hydrolysis with ATP alone (Table 5). The

Table 5. *Bivalent cation-activated ATPase in intact granules and the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone*

ATPase activities in freshly-prepared granules were determined spectrophotometrically under iso-osmotic incubation conditions using maleic acid/Tris buffer (pH 7) (see the Experimental section). Each tabulated value is the mean $\pm$ S.E.M. of results obtained with six different granule preparations. The statistical significance of differences refers to the effect of adding the protonophore (*, $P < 0.001$; **, $P < 0.02$; ***, $P < 0.01$). Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Addition	P_i formation ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	
	Control	Plus FCCP
ATP alone	8.5 ± 1.5	9.5 ± 2.1
ATP plus CDTA	2.9 ± 1.3	3.8 ± 0.8
Mg^{2+} -ATP	63.4 ± 11.9	$136.2 \pm 15.3^{**}$
Mn^{2+} -ATP	53.9 ± 8.9	$134.3 \pm 10.7^*$
Co^{2+} -ATP	38.7 ± 5.7	$73.4 \pm 10.9^{***}$
Ca^{2+} -ATP	26.8 ± 4.3	29.1 ± 2.7

addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to the media had no effect in the absence of exogenous bivalent cations but increased ATP hydrolysis by approximately 2-fold in the presence of Mg^{2+} , Mn^{2+} or Co^{2+} . Activity supported by Ca^{2+} , however, was not affected by this compound.

The basal rate of ATP hydrolysis and the magnitude of the response to bivalent cation addition observed with intact granules was similar to that in freeze/thawed preparations (Table 4). In the former circumstance, however, no precautions were taken to avoid contamination of the assay media with endogenous bivalent cations (potentially $40\text{--}50\text{ }\mu\text{M}$ of Ca^{2+} or Mg^{2+}). It was thus evident that Mg^{2+} and Ca^{2+} in intact granules are localized internally and that the granule membrane has a low permeability to

Table 4. *The effects of tributyltin and NN'-dicyclohexylcarbodi-imide on bivalent cation-activated ATPase in freeze/thawed granules*

Spectrophotometric assays were performed using Tris/HCl buffer (pH 7.5) (see the Experimental section). Each tabulated value is the mean $\pm$ S.E.M. of results obtained with different granule preparations; the numbers of these are shown in parentheses. The statistical significance of differences refers to the effect of adding the inhibitor (*, $P < 0.001$; **, $P < 0.02$; ***, $P < 0.05$). Abbreviation: DCCD, NN' -dicyclohexylcarbodi-imide.

Addition	P_i formation ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)		
	Control	Plus tributyltin	Plus DCCD
ATP alone	13.5 ± 2.5 (12)	<1.0 (3)**	4.2 ± 0.3 (3)
CDTA plus ATP	4.2 ± 1.3 (9)	1.9 ± 0.1 (3)*	<1.0 (3)
Mg^{2+} -ATP	88.4 ± 6.4 (17)	18.5 ± 3.4 (5)*	19.5 ± 3.7 (5)*
Mn^{2+} -ATP	72.1 ± 11.0 (10)	15.8 ± 0.6 (3)**	9.6 ± 4.5 (3)**
Co^{2+} -ATP	31.8 ± 6.3 (4)	5.7 ± 2.4 (3)**	7.8 ± 0.4 (3)**
Ca^{2+} -ATP	35.4 ± 3.4 (16)	25.9 ± 2.3 (5)	23.7 ± 2.3 (4)

Table 6. *Bivalent cation effects on the chemiosmotic properties of intact granules*

The uptake of [^{14}C]methylamine and thio[^{14}C]cyanate by freshly-prepared granules is expressed as described in the legend to Table 3. Each tabulated value is the mean $\pm$ S.E.M. of results obtained with three to five different granule preparations: n represents the number of separate incubations performed. The statistical significance of differences refers in each case to the control incubation performed without bivalent cations at the same nucleotide concentration (*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$).

Addition	n	Methylamine uptake ratio	Thiocyanate uptake ratio	ΔpH	$\Delta\psi$ (mV)	$\Delta\mu_{\text{H}^+}$ (mV)
None	11	18.81 ± 1.36	1.56 ± 0.45	1.27	11.6	88.0
Mg^{2+}	11	13.49 ± 0.88	1.02 ± 0.10	1.13	0.5	68.3
Mn^{2+}	4	13.46 ± 0.41	1.14 ± 0.10	1.13	4.4	71.2
Co^{2+}	4	14.98 ± 0.54	1.51 ± 0.35	1.18	10.7	81.2
Ca^{2+}	4	$13.10 \pm 0.89^{***}$	1.14 ± 0.18	1.12	3.4	70.5
ATP	11	15.65 ± 2.51	2.44 ± 0.22	1.19	23.2	94.9
Mg^{2+}ATP	11	16.55 ± 1.83	$7.74 \pm 1.10^*$	1.22	53.3	126.4
Mn^{2+}ATP	11	18.53 ± 1.96	$8.39 \pm 0.85^*$	1.27	55.4	131.4
Co^{2+}ATP	8	15.50 ± 2.09	$7.06 \pm 0.55^*$	1.19	50.9	122.3
Ca^{2+}ATP	11	15.85 ± 2.45	2.74 ± 0.18	1.20	26.2	98.2

these ions, a conclusion that is consistent with recent localization studies (J. C. Hutton & M. Peshavaria, unpublished work). It follows that, since the Mg^{2+}ATP complex was evidently the substrate for the proton translocase, the catalytic site of the enzyme is accessible only on the external face of the granule membrane.

Bivalent cation effects on the chemiosmotic properties of granules. ATP addition by itself, or bivalent cations in the absence of exogenous ATP, did not produce marked changes in the ΔpH , $\Delta\psi$ or $\Delta\mu_{\text{H}^+}$ across the granule membrane (Table 6). Mg^{2+} , Mn^{2+} and Co^{2+} added in conjunction with ATP, however, increased markedly the $\Delta\psi$ and $\Delta\mu_{\text{H}^+}$ but did not affect the ΔpH . Ca^{2+} in the presence of ATP was without this effect, indicating that Ca^{2+} -activated ATP hydrolysis was not coupled to proton translocation. This was consistent with the above findings that the Ca^{2+} -activated ATPase of intact granules was not stimulated by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 5) or inhibited in freeze/thawed granule preparations by either tributyltin or *NN'*-dicyclohexylcarbodi-imide (Table 4).

The relationship between changes in the $\Delta\psi$ across the granule membrane and the rates of ATP hydrolysis by intact granules supported by different bivalent cations was indistinguishable from that observed with different nucleotides in the presence of Mg^{2+} (Fig. 2a). It was also apparent that the carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone- and tributyltin-sensitive ATPase activities under these conditions were closely correlated (Fig. 2b). This further supported the proposition that nucleotide-driven proton translocation is responsible for the changes seen in the $\Delta\psi$ and

$\Delta\mu_{\text{H}^+}$ (Hutton, 1982). These findings suggested that the stoichiometry of H^+ translocation to NTP hydrolysis was identical in each case, which would be consistent with the functioning of a single enzymic species. The indicated specificity was: $\text{Mg}^{2+}\text{ATP} = \text{Mn}^{2+}\text{ATP} > \text{Co}^{2+}\text{ATP} > \text{Mg}^{2+}\text{GTP} > \text{Mg}^{2+}\text{ITP} > \text{Mg}^{2+}\text{CTP}$.

Discussion

The vectorial transport of H^+ coupled to ATP hydrolysis occurs in a wide variety of biological membrane systems in both eukaryotes and prokaryotes. The enzymes responsible show many common features including similarities in subunit composition, possession of common antigenic determinants and sensitivity to inhibition by *NN'*-dicyclohexylcarbodi-imide (see Downie *et al.*, 1979). The inhibitory effect of the latter compound results from high affinity binding within the proton channel to a protein that is possibly a ubiquitous component of these enzymes (Beechey *et al.*, 1967). The inhibitory effects of trialkyltin compounds on several proton translocases reside also in this region (Dawson & Selwyn, 1975), which would account, in the present circumstance, for the parallel inhibitory effects of *NN'*-dicyclohexylcarbodi-imide and tributyltin on the insulin-granule ATPase activity with different bivalent cations. Other effects of tributyltin resulting from its ability to catalyse a Cl^-/OH^- exchange (Stockdale *et al.*, 1970) would not be expected to affect nucleotide hydrolysis in the assays presently used, since these were performed on osmotically-disrupted granule preparations.

It was apparent that neither *NN'*-dicyclohexylcarbodi-imide nor tributyltin entirely suppressed

Fig. 2. Relationships between nucleotide and bivalent cation effects on enzymic activity and changes in the $\Delta\psi$. Data were taken from Tables 1–6. In (a), changes in the $\Delta\psi$ are shown in relation to rates of P_i formation by intact granules. In (b), the relationship is shown between the decrement in enzymic activity produced by tributyltin in freeze/thawed preparations and the increment produced in intact granules by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. $\bullet$, *trans*-1,2-Diaminocyclohexanetetra-acetic acid + ATP. All other symbols are labelled in the Figure. Abbreviations: FCCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NTPase, nucleoside triphosphatase.

rates of hydrolysis in the presence of these inhibitors were of a similar magnitude, which indicated that the same enzyme might be involved but that the rate-limiting factor was one of nucleotide hydrolysis rather than proton translocation. Such a hypothesis would explain, for example, why Mg^{2+} -CTP hydrolysis, though not markedly affected by tributyltin and *p*-trifluoromethoxyphenylhydrazone, could nevertheless support a change in the $\Delta\psi$.

The present findings support a previous suggestion based on inhibitor experiments that the insulin granule proton translocase activity differs from that in mitochondria, but is possibly identical with the adrenal chromaffin granule enzyme (Hutton & Peshavaria, 1982). The nucleotide and bivalent cation specificity deduced for the insulin granule enzyme coincided with that of amine transport in intact isolated chromaffin granules (Kirshner, 1962), a process that is linked to proton translocation (see Njus *et al.*, 1981). The ability of Mn^{2+} and Co^{2+} , as well as Mg^{2+} , to support uncoupler-sensitive ATP hydrolysis in chromaffin granules (Bashford *et al.*, 1975) also agreed with our observations here.

Similarities were evident between the insulin granule proton translocase and anion-sensitive ATPases described in microsomal fractions from gastric mucosal (Blum *et al.*, 1971) and pancreatic tissue (Simon & Thomas, 1972) and in a 'secretory granule' fraction prepared from frozen adenohipophyses (Lorenson *et al.*, 1981). The microsomal enzymes were originally proposed as anion-transporting proteins but recently it has been suggested that they are merely artefacts of mitochondrial disruption (Schuurmans Stekhoven & Bonting, 1981). Unlike the insulin granule proton translocase activity, the pancreatic microsomal enzyme can hydrolyse ATP in the presence of Zn^{2+} , a property that is shared by the mitochondrial ATPase (Selwyn, 1967).

The Zn^{2+} -induced inhibition of the insulin granule bivalent cation-supported ATPase activity that was observed has also been reported with a proton-translocating Mg^{2+} -dependent ATPase found in vacuolar membranes of the yeast *Saccharomyces cerevisiae* (Kakinuma *et al.*, 1981). The nucleotide and bivalent cation specificity of the yeast enzyme is identical with that of the insulin granule proton translocase activity, it has a similar K_m for ATP and is likewise insensitive to inhibition by oligomycin and vanadate. Proton translocation in the yeast vacuole, as in the chromaffin granule, can be coupled to the inwardly-directed transport of a cationic species, namely, arginine (Ohsumi & Anraku, 1981). It would seem that this enzyme and those of mammalian secretory granules constitute a class of proteins characteristically associated with intracellular storage vesicles in eukaryotic cells. Their presence here may be a reflection either of a

nucleotide hydrolysis in the presence of bivalent cations. This was conceivably related to the presence of other nucleoside triphosphatases. However, the

common evolutionary origin (see Hales, 1978) or a common function.

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