The internal pH and membrane potential of the insulin-secretory granule

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The membrane potential ($\Delta\Psi$) and the pH gradient (ΔpH) across the membrane of the insulin-secretory granule were determined in studies *in vitro* from the uptake of the permeant anion thio[^{14}C]cyanate or the permeant base [^{14}C]methylamine. Freshly prepared granules incubated in iso-osmotic medium containing sucrose and low concentrations of buffer salts exhibited an acidic internal pH and a $\Delta\Psi$ positive inside. Addition of MgATP^{2-} under these conditions did not alter the ΔpH , but produced a marked increase in the $\Delta\Psi$. Conversely, when a permeant anion was also included, ATP produced a marked increase in the ΔpH and a lesser increment in the $\Delta\Psi$. NH_4^+ salts reduced the ΔpH across granule membranes. In the presence of ATP this effect was accompanied by a reciprocal increase in the $\Delta\Psi$. A similar reciprocity was evident when nigericin was added together with K^+ or on decreasing the medium pH, suggesting that these gradients were linked by a common electrogenic process. The effects of ATP were reversed by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, the combination of valinomycin, nigericin and K^+ , and by the Mg^{2+} -dependent ATPase inhibitor tributyltin. Uptakes of ^{14}C -labelled tracer molecules were also markedly reduced by cryogenic disruption of the granule membrane or hypo-osmotic incubation conditions. These results were readily interpreted within a chemiosmotic hypothesis, which proposed that the insulin granules possess an inwardly-directed electrogenic proton-translocating Mg^{2+} -dependent ATPase with the additional postulate that the membrane has a low proton permeability. The intragranular pH was estimated as being between 5 and 6 *in vivo*. Such a value corresponds to optimal conditions for the crystallization of zinc–insulin hexamers. Several other functions related to chemiosmotic processes within insulin granules, however, may be envisaged.

The maintenance of pH gradients across intracellular membranes of mammalian cells, be they linked to respiration, ATP hydrolysis or a Donnan equilibrium, appear to relate to diverse physiological functions. Proton gradients in mitochondria are associated with energy transduction, ATP synthesis and ion transport; in the lysosome the acidic interior matches the acidic pH optima of many of its constituent enzymes; in the neurosecretory vesicle of the neurohypophysis the internal pH corresponds to the maximal stability of the hormone–neurophysin complex, and in the chromaffin granule of the adrenal medulla the pH gradient is operative in the uptake and storage of its secreted product (for review, see Roos & Boron, 1981; Russell & Holz, 1981).

Viewed within this context, the observation that

insulin-secretory granules possess a proton-translocating Mg^{2+} -dependent ATPase activity (Hutton & Peshavaria, 1982) may have a particular bearing on the constitution and function of these organelles. The present paper documents the ΔpH and $\Delta\Psi$ across the membrane of the insulin-secretory granule as determined from the uptake of a radioisotopically-labelled permeant base or anion *in vitro*. The objectives of these studies were to estimate the prevailing internal pH of the granule *in vivo* and to test further the chemiosmotic model proposed for the function of the proton-translocating Mg^{2+} -dependent ATPase activity in the granule membrane.

Experimental

Materials

Insulin-secretory granules were prepared from a transplantable rat islet-cell tumour (Chick *et al.*, 1977) by a combination of differential centrifuga-

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

tion and density-gradient centrifugation on Percoll gradients as previously described (Hutton & Peshavaria, 1982). These were suspended at 2–5 mg/ml at 4°C and used within 4 h.

[¹⁴C]Methylamine hydrochloride (sp. radioactivity 60 Ci/mol), potassium thio[¹⁴C]cyanate (sp. radioactivity 60 Ci/mol), inulin[¹⁴C]carboxylic acid (sp. radioactivity 6 Ci/mol) and ³H₂O (5 Ci/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Nigericin was a gift from Roche Products, Welwyn Garden City, Herts., U.K., tributyltin was obtained from Ralph N. Emmanuel, Alperton, Middx., U.K., and other chemicals from Sigma (London) U.K., Kingston upon Thames, Surrey, U.K., or BDH, Poole, Dorset, U.K.

Determination of ΔpH and $\Delta\Psi$

Radioisotope distribution experiments were performed in medium containing (final concentration) 0.25 M-sucrose, 0.5 mM-EGTA, 1 mM-MgSO₄ and 25 mM-4-morpholine-ethanesulphonic acid adjusted to the desired pH with Tris base. ATP (final concentration 3.3 mM) was added with an equimolar quantity of MgSO₄ from a solution adjusted to the same pH with Tris base. Other additions are described in the Results section. Where relevant, control incubations contained the solvent dimethyl sulphoxide, which by itself did not affect any parameter determined in the present paper. Under the conditions used, less than 20% of the available ATP was hydrolysed and the medium pH at completion of the incubation was found to differ by less than 0.05 units from its initial value.

For each experimental condition duplicate or triplicate determinations were made in medium containing 5 μ Ci of ³H₂O/ml combined with one of the following radioisotopes; 1 μ Ci of [¹⁴C]methylamine/ml (ΔpH), 1 μ Ci of thio[¹⁴C]cyanate/ml ($\Delta\Psi$) or 2.5 μ Ci of inulin[¹⁴C]carboxylic acid/ml (extragranular water space). Granules suspended in 120 μ l of medium at about 0.4 mg of protein/ml were incubated at 37°C for 20 min in a 1 ml capacity Eppendorf microfuge tube (W. Sarstedt, Leicester, U.K.) and then pelleted by centrifugation at room temperature for 4 min at 9500 g. Water-saturated dibutyl phthalate (300 μ l) was added to each tube and a further centrifugation for 10 s performed to partition the aqueous medium from the granule pellet. The tube tip was removed with a scalpel blade, transferred to a scintillation vial containing 0.5 ml of water and 5 ml of a Triton X-100/toluene-based scintillation cocktail (mixture T; Hopkin and Williams, Chadwell Heath, Essex, U.K.) and the radioisotopic composition determined by dual-channel liquid-scintillation spectrometry (Packard Instruments, Downers Grove, IL, U.S.A.). Control incubations contained all constituents except the granule suspension and were treated in an identical

manner. The radioactivities found in these controls were less than 10% of those in the presence of granules and were subtracted from the experimental values.

Calculations

The internal water space of the granule was calculated from the difference in the apparent spaces of distribution of ³H₂O and inulin[¹⁴C]carboxylic acid divided by the pellet-protein content. This was not found to vary significantly with any experimental condition using intact granules. The mean intragranular space was $2.00 \pm 0.10 \mu\text{l/mg}$ of protein ($n = 189$) and represented between 30 and 60% of the total water space found in each pellet.

The $\Delta\Psi$ and the ΔpH across the granule membrane were calculated from the following formulae:

$$\Delta\Psi = 60 \cdot \log \left(\frac{R_t - R_i}{1 - R_i} \right) \quad (1)$$

and

$$\Delta pH = -\log \left(\frac{R_m - R_i}{1 - R_i} \right) \quad (2)$$

where R_t , R_m and R_i were the ratios of radioactivity from thio[¹⁴C]cyanate, [¹⁴C]methylamine and inulin[¹⁴C]carboxylic acid respectively to that of ³H₂O found in the same pellet. The pH gradient could be converted into units identical with those of the membrane potential by its product by -60 , which thus allowed the calculation of the proton potential ($\Delta\mu H^+$) according to the equation:

$$\Delta\mu H^+ = -60 \cdot \Delta pH + \Delta\Psi (\text{mV})$$

The theoretical and practical aspects of these methods and calculations have been extensively reviewed (Rottenberg, 1979; Roos & Boron, 1981).

Protein was determined by the method of Lowry *et al.* (1951) after precipitation of the samples with 5% (w/v) trichloroacetic acid with bovine serum albumin (fraction V; Sigma) as standard.

The statistical significance of differences of means was estimated by Student's *t* test for unpaired data. Since ΔpH and $\Delta\Psi$ measurements did not follow a Gaussian distribution, statistical analyses were conducted on the ratios of intragranular to extragranular concentrations of [¹⁴C]methylamine or of thio[¹⁴C]cyanate. These ratios are represented by the terms in parentheses in the above equations.

Results

Insulin granules incubated in the presence of MgATP²⁻ accumulated [¹⁴C]methylamine or thio[¹⁴C]cyanate 5–10-fold relative to the initial concentration of these isotopes in the surrounding medium. Examination of the time course of uptake

showed that thio[^{14}C]cyanate had equilibrated within 5 min and did not change within 30 min. [^{14}C]-Methylamine uptake, however, was slower, reaching a steady state only after 15 min. Markers of the total volume (3H_2O) and extragranular water space (inulin[^{14}C]carboxylic acid) had equilibrated by 5 min and were unchanged thereafter.

Granules that were subjected to four cycles of freeze-thawing or incubated under hypo-osmotic conditions accumulated much smaller amounts of [^{14}C]methylamine and thio[^{14}C]cyanate than controls (Table 1). This was apparent whether results were expressed in terms of the ratio of intragranular to extragranular concentration or as the specific radioactivity of the pelleted material. In control incubations 74% of the protein added initially was recovered in the final pellet. After freeze-thaw treatment and hypo-osmotic incubation the recovery was reduced to 52% and 54% respectively. These differences, however, were not of a sufficient magnitude to account for the present results. These findings suggested that the majority of the [^{14}C]-methylamine and thio[^{14}C]cyanate accumulation by intact granules was attributable to the presence of pH and potential differences across sealed membranes. The results obtained under hypo-osmotic conditions indicated that the maximum contribution of a non-specific binding component to the total uptake was less than 10%. The relative efficacies of cryogenic disruption and hypo-osmotic treatment to diminish granule [^{14}C]methylamine or thio[^{14}C]cyanate uptakes matched their relative capacities to increase Mg^{2+} -dependent ATPase activity in granules (Hutton & Peshavaria, 1982).

Radioisotopic uptake studies performed with 5 or 25 μM concentrations of the tracer molecules gave identical intragranular/extragranular concentration ratios with those observed in control incubations at 16 μM . This suggested that these compounds did not alter the electrochemical gradients they had been used to monitor.

*Effects of ATP, tributyltin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone*

Granules incubated without ATP in medium containing sucrose and low concentrations of buffer salts maintained a ΔpH of approximately unity, acidic inside, across their membranes (Table 2). A $\Delta\Psi$ of about 20 mV was also evident, positive with regard to the interior.

The addition of $MgATP^{2-}$ to the incubation medium did not significantly affect the ΔpH under these conditions but resulted in a substantial increase in the $\Delta\Psi$ and hence the $\Delta\mu H^+$ ($P < 0.005$).

Tributyltin did not significantly affect either the ΔpH or $\Delta\Psi$ in granules incubated without ATP but prevented the ATP-induced increment in $\Delta\Psi$. The effect might be attributed either to inhibition of the granule Mg^{2+} -dependent ATPase activity (Hutton & Peshavaria, 1982) or to an anion/ OH^- exchange that this compound can catalyse (Stockdale *et al.*, 1970). Such anions would need to be derived from the granule itself since no permeant anion was added in the present circumstance. Also in the latter case a substantial effect on the ΔpH would be expected in both the presence and absence of ATP.

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which releases the membrane-imposed control of ATP hydrolysis in intact granules (Hutton & Peshavaria, 1982) also prevented the ATP-induced increment in the $\Delta\Psi$ ($P < 0.001$) and additionally caused a decrease in the ΔpH both in the presence ($P < 0.005$) or absence ($P < 0.005$) of ATP. Such effects were consistent with its capacity as a protonophore to discharge electrochemical gradients resulting from proton translocation.

The low or negative $\Delta\Psi$ values observed in incubations containing either tributyltin or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone could not be accurately determined by the present technique based on thio[^{14}C]cyanate uptake. The lower limit of reliable estimation was approx. +10 mV; this

Table 1. *Effect of membrane perturbation on [^{14}C]methylamine and thio[^{14}C]cyanate uptake by insulin-secreting granules*

Radioisotopic uptake studies were performed in the presence of ATP at pH 7 as described in the Experimental section. Hypo-osmotic disruption was effected by omitting sucrose from assay media, cryogenic disruption by subjecting granule suspensions (5 mg/ml) in sucrose-containing media to four cycles of freeze-thawing ($-20^\circ C/37^\circ C$) over a 30 min period and then incubating them under conditions identical with the control. Each tabulated value is the mean $\pm$ S.E.M. of results obtained with two to five different granule preparations; the numbers of separate incubations performed are shown in parentheses. The statistical significance of differences refers in each case to the control value (* $P < 0.001$).

Treatment	Total water space ($\mu l/mg$)	Extragranular space ($\mu l/mg$)	Intragranular [^{14}C]methylamine (nmol/mg)	Intragranular thio[^{14}C]cyanate (nmol/mg)
Control	3.73 ± 0.11 (11)	1.95 ± 0.11 (10)	302 ± 36 (5)	145 ± 12 (5)
Cryogenic disruption	2.82 ± 0.06 (7)*	2.07 ± 0.06 (8)	55 ± 1 (4)*	38 ± 10 (4)*
Osmotic disruption	2.45 ± 0.16 (12)*	0.96 ± 0.13 (3)*	32 ± 6 (4)*	12 ± 2 (4)*

Table 2. *Effect of ATP, tributyltin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on the ΔpH and $\Delta \Psi$ across the insulin granule membrane*

Radioisotope uptake measurements were performed at pH 7 as described in the Experimental section using tributyltin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at a final concentration of 2.5 μM . [^{14}C]Methylamine or thio[^{14}C]cyanate uptakes were in each case expressed as the ratio of their intragranular to medium concentrations. Each tabulated value is the mean $\pm$ S.E.M. of results obtained from two to seven different granule preparations; the number of separate incubations performed is shown in parentheses. The statistical significance of differences shown (* $P < 0.001$; ** $P < 0.005$) relate to the control incubation performed at the same ATP concentration.

Addition	Methylamine- uptake ratio	Thiocyanate- uptake ratio	ΔpH	$\Delta \Psi$ (mV)	$\Delta \mu H^+$ (mV)
None	9.19 $\pm$ 0.69 (10)	2.11 $\pm$ 0.77 (9)	0.96	19.4	77.3
Tributyltin	7.80 $\pm$ 0.54 (5)	0.49 $\pm$ 0.16 (5)	0.89	<10.0	—
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	7.05 $\pm$ 0.68 (11)	1.28 $\pm$ 0.78 (11)	0.85	<10.0	—
ATP	7.97 $\pm$ 0.71 (18)	5.53 $\pm$ 0.64 (19)	0.90	44.5	98.7
ATP + tributyltin	6.66 $\pm$ 0.19 (5)	0.44 $\pm$ 0.10 (5)*	0.82	<10.0	—
ATP + carbonyl cyanide cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	5.47 $\pm$ 0.40 (20)**	0.93 $\pm$ 0.25 (19)*	0.74	<10.0	—

Table 3. *Effect of anions on the ΔpH and $\Delta \Psi$ across the insulin granule membrane*

Radioisotope uptake measurements were performed at pH 7 as described in the Experimental section using K_2SO_4 , KCl and KI at concentrations of 25, 50 and 50 mM respectively. [^{14}C]Methylamine and thio[^{14}C]cyanate uptakes were in each case calculated as the ratio of their intragranular to medium concentration. Each tabulated value is the mean $\pm$ S.E.M. of results obtained with two to six different granule preparations; the number of separate incubations performed is shown in parentheses. The statistical significance of differences shown (* $P < 0.001$; ** $P < 0.01$) relate to the effect of ATP addition.

Addition	Methylamine- uptake ratio	Thiocyanate- uptake ratio	ΔpH	$\Delta \Psi$ (mV)	$\Delta \mu H^+$ (mV)
None	14.03 $\pm$ 0.78 (13)	3.81 $\pm$ 1.20 (13)	1.15	34.8	103.7
ATP	12.44 $\pm$ 0.67 (17)	10.00 $\pm$ 0.78 (17)*	1.09	60.0	125.7
K_2SO_4	9.19 $\pm$ 0.64 (12)	5.78 $\pm$ 0.75 (12)	0.96	45.7	103.5
K_2SO_4 + ATP	10.67 $\pm$ 0.70 (16)	8.81 $\pm$ 0.67 (15)**	1.03	56.7	118.4
KCl	9.39 $\pm$ 0.95 (12)	4.81 $\pm$ 1.20 (12)	0.97	40.9	99.3
KCl + ATP	17.92 $\pm$ 1.64 (15)*	7.36 $\pm$ 0.67 (15)	1.25	52.0	127.2
KI	7.03 $\pm$ 0.70 (5)	0.83 $\pm$ 0.47 (5)	0.85	<10.0	—
KI + ATP	20.61 $\pm$ 3.11 (5)*	1.78 $\pm$ 0.58 (5)	1.31	15.0	93.8

represented a 20% increase in the space of distribution of the tracer over that of 3H_2O in the pelleted material and was at a level where small variation in the extragranular water space and non-specific binding effects could affect calculations.

Response of the ΔpH and $\Delta \Psi$ to changes in medium pH

Decreasing the medium pH in the range of 7.2 to 5.9 greatly increased the $\Delta \Psi$ and decreased the ΔpH across granule membranes in the presence of ATP. The changes in pH expressed in mV was reciprocated by the change in the $\Delta \Psi$, thus suggesting that the ΔpH and $\Delta \Psi$ were linked by a common electrogenic process.

In the absence of ATP the ΔpH also decreased as a function of decreasing external pH. The response of the $\Delta \Psi$ under these conditions, however, could not be reliably ascertained because of the above-mentioned technical limitations.

Effects of different K^+ salts

In the absence of ATP the addition of K^+ to the incubation medium, whether in the form of K_2SO_4 , KCl or KI, resulted in a decrease in the ΔpH across the granule membrane ($P < 0.001$) but did not affect the $\Delta \Psi$ in a consistent manner (Table 3).

ATP induced an increase in the $\Delta \Psi$ and no change in the ΔpH in the presence of K_2SO_4 similar to its effects in medium containing sucrose alone. By contrast, when the anion was Cl^- or I^- , a large increase in the ΔpH was observed on ATP addition. This was accompanied by a smaller increment in the $\Delta \Psi$ than that observed in the presence of sucrose alone, thus the overall ATP-induced change in $\Delta \mu H^+$ was not substantially altered.

Previous studies suggested that the insulin-granule membrane is more permeable to Cl^- and I^- than to SO_4^{2-} (Hutton & Peshavaria, 1982). Inwardly directed ATP-driven proton movements, if accompanied by Cl^- or I^- permeation, would lead to the

electroneutral accumulation of a strong acid within the granule interior, thus accounting for the present observations.

Secretory granules obtained from the neurohypophysis or adrenal medulla release their internal contents after incubation in the presence of KCl and ATP (Poisner & Trifaró, 1967; Poisner & Douglas, 1968). Insulin granules under similar conditions are reportedly stable (Howell *et al.*, 1969). Granule lysis did not occur in the present experiments at least as judged from the finding that the internal volume of the granule was not affected by different K^+ salts or ATP (J. C. Hutton, unpublished work). It should be noted also that the present media were hyperosmolar and contained lower concentrations of the various K^+ salts than those used by the above authors.

Effects of NH_4^+ salts

The addition of either $(NH_4)_2SO_4$ or NH_4Cl to granules incubated without ATP markedly reduced the ΔpH across their membranes without significantly altering the $\Delta\Psi$ (Table 4). Similar effects of NH_4^+ on the ΔpH have been inferred from examination of 9-aminoacridine fluorescence quenching by granule-enriched fractions from pancreatic islets (Abrahamsson & Glyfe, 1980).

In the presence of ATP, these NH_4^+ salts still reduced the ΔpH across the granule membrane; however, the ATP-induced increment in $\Delta\Psi$ was far greater. The $\Delta\mu H^+$ nevertheless was not substantially affected.

$(NH_4)_2SO_4$ was more effective than NH_4Cl in producing these changes, at least when comparison was made with results obtained without added salts (Table 4). Compared with experiments performed at similar concentrations of their respective K^+ salts (Table 3) the effect of NH_4Cl on the ΔpH was actually more pronounced than that of $(NH_4)_2SO_4$, the converse being true for the effect on the $\Delta\Psi$.

Investigation of the concentration dependency of the ΔpH changes induced by $(NH_4)_2SO_4$ in the absence of ATP revealed significant effects at concentrations as low as 10 mM with virtual abolition of the ΔpH at 100 mM (J. C. Hutton, unpublished work).

These effects of NH_4^+ were readily interpretable within the proposed chemiosmotic model. The permeation of the granule by the uncharged NH_3 molecule would be expected to neutralize the ΔpH across the membrane whether ATP is present or not. Proton translocation induced by ATP would be accompanied by the inward flux of NH_4^+ and hence the expression of the $\Delta\mu H^+$ principally as its $\Delta\Psi$ component. The concomitant presence of a permeant anion would exaggerate the change in ΔpH induced by NH_4^+ while reducing the $\Delta\Psi$.

Effects of nigericin and valinomycin

The electrogenic K^+ ionophore valinomycin added to granules incubated with K_2SO_4 and ATP did not significantly affect either the ΔpH or the $\Delta\Psi$ across their membranes (Table 5). The K^+ content of freshly isolated granules was 30–40 mg ion/mg of protein, i.e. approx. 15–20 mg ion/litre of intragranular water (J. C. Hutton, unpublished work). The extragranular K^+ concentration being 25 mg ion/litre, the contribution of the transmembrane K^+ gradient to the $\Delta\Psi$ in the presence of valinomycin would have been minor. Studies of chromaffin-granule ghosts in any case indicate that any K^+ /valinomycin-induced potential (Njus & Radda, 1979) may have decayed during the course of the subsequent incubation.

Nigericin, which under present conditions would catalyse an H^+/K^+ exchange across the granule membrane, produced a large decrease in the ΔpH in the presence of ATP while promoting a reciprocal increase in the $\Delta\Psi$. These findings, which were equivalent to the effects of NH_4^+ , can be accounted

Table 4. *Effect of NH_4^+ salts on the ΔpH and $\Delta\Psi$ across the insulin-granule membrane*

Radioisotope uptake measurements were performed at pH 7 as described in the Experimental section using $(NH_4)_2SO_4$ and NH_4Cl at concentrations of 20 and 40 mM respectively. [^{14}C]Methylamine and thio[^{14}C]cyanate uptakes were in each case calculated as the ratio of their intragranular to medium concentrations. Each tabulated value is the mean $\pm$ S.E.M. of results obtained in two to seven different granule preparations; the number of separate incubations performed is shown in parentheses. The statistical significance of differences shown (* $P < 0.001$) relates to the effect of adding the designated salt.

Addition	Methylamine- uptake ratio	Thiocyanate- uptake ratio	ΔpH	$\Delta\Psi$ (mV)	$\Delta\mu H^+$ (mV)
None	17.86 ± 1.81 (20)	1.78 ± 0.58 (20)	1.25	15.0	90.0
$(NH_4)_2SO_4$	4.00 ± 0.72 (10)*	1.83 ± 0.25 (10)	0.60	15.8	51.8
NH_4Cl	4.42 ± 0.39 (4)*	0.42 ± 0.42 (4)	0.65	<10.0	—
ATP	15.39 ± 1.45 (20)	6.78 ± 0.92 (20)	1.19	49.9	121.1
ATP + $(NH_4)_2SO_4$	4.28 ± 0.50 (20)*	15.00 ± 0.50 (20)*	0.63	70.6	108.4
ATP + NH_4Cl	7.33 ± 0.89 (4)*	9.58 ± 1.36 (4)	0.87	58.8	110.8

Table 5. *Effect of valinomycin and nigericin on the ΔpH and $\Delta \Psi$ across the insulin-granule membrane*

Radioisotope uptake measurements were performed at pH 7 as described in the Experimental section using 12.5 mM- K_2SO_4 , 0.25 μM -valinomycin and 0.1 μM -nigericin. [^{14}C]Methylamine and thio[^{14}C]cyanate uptakes were in each case calculated as the ratio of their intragranular to medium concentrations. Each tabulated value is the mean $\pm$ S.E.M. of results obtained with six different granule preparations; the number of separate incubations performed is shown in parentheses. The statistical significance of differences (* $P < 0.001$; ** $P < 0.005$; *** $P < 0.01$; **** $P < 0.05$) refers in each case to incubations without the ionophores at the same ATP concentration.

	Methylamine- uptake ratio	Thiocyanate- uptake ratio	ΔpH	$\Delta \Psi$ (mV)	$\Delta \mu H^+$ (mV)
ATP/ K^+	10.56 $\pm$ 2.17 (18)	9.75 $\pm$ 0.67 (16)	1.02	59.3	120.7
ATP/ K^+ /valinomycin	12.64 $\pm$ 2.89 (14)	8.72 $\pm$ 2.89 (14)	1.10	56.4	122.5
ATP/ K^+ /nigericin	3.81 $\pm$ 0.36 (8)**	19.61 $\pm$ 0.36 (8)*	0.58	77.6	112.4
ATP/ K^+ /valinomycin/nigericin	3.81 $\pm$ 0.75 (8)***	1.75 $\pm$ 0.36 (8)*	0.58	14.5	49.4
ATP/valinomycin/nigericin	10.86 $\pm$ 0.72 (4)	8.81 $\pm$ 0.72 (4)	1.04	56.7	118.9
K^+	11.20 $\pm$ 0.70 (5)	5.82 $\pm$ 0.22 (5)	1.05	45.9	108.8
K^+ /valinomycin/nigericin	4.89 $\pm$ 1.07 (4)*	1.92 $\pm$ 0.75 (4)*	0.69	17.0	58.4

for by the proposed dependence of these gradients on a proton-translocating ATPase with the further postulate that the granule membrane has a relatively low permeability to K^+ . Evidence for the latter was obtained from studies of Mg^{2+} -dependent ATPase activity in intact granules (Hutton & Peshavaria, 1982).

The combination of nigericin and valinomycin reduced both the ΔpH and the $\Delta \Psi$ across the granule membrane in the presence of ATP, an effect that was dependent on the simultaneous presence of K_2SO_4 . Under such conditions valinomycin could discharge the electrochemical K^+ gradient produced by the proton-translocating ATPase acting in concert with nigericin. The concomitant decrease in both the ΔpH and $\Delta \Psi$ under these conditions suggested that the thermodynamic constraint to vectorial proton translocation was removed. Consistent with this was the finding that the membrane-imposed control of ATP hydrolysis was markedly reduced under such incubation conditions (Hutton & Peshavaria, 1982).

The combination of valinomycin, nigericin and K^+ also significantly reduced the ΔpH and $\Delta \Psi$ across the membrane of granules incubated without ATP. In achieving a reduction in the ΔpH under these conditions it was more effective than carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 2).

Discussion

Proton-translocating Mg^{2+} -dependent ATPase activity in insulin granules

The effects of ATP on the ΔpH and $\Delta \Psi$ across the membrane of the insulin granule under a variety of experimental conditions were entirely consistent with the chemiosmotic model formulated previously (Hutton & Peshavaria, 1982). This suggested that

secretory-granule membranes possess an inwardly directed proton-translocating Mg^{2+} -dependent ATPase activity and low proton permeability. Experimental treatments that disrupted the granule (Table 1) or increased its permeability to protons (Tables 2 and 5) reduced its capacity to respond to ATP by changes in ΔpH or $\Delta \Psi$. Inhibition of Mg^{2+} -dependent ATPase activity by tributyltin had a similar effect (Table 2). Results obtained after modification of the medium pH (Fig. 1), inclusion of nigericin (Table 5), a permeant base (Table 4) or permeant anion (Table 3) demonstrated the ΔpH and were linked to a common electrogenic process in the presence of ATP. Indeed incubation conditions could be chosen that would couple ATP hydrolysis by the granule to either the ΔpH or $\Delta \Psi$ component of the protonmotive force.

The intragranular pH in vivo

Freshly isolated granules incubated in the absence of ATP at pH 7 had an internal pH of 6 or below. Extrapolation of data obtained at different medium-pH values (Fig. 1) to a null ΔpH across the membrane suggested a minimum value of 5 to 5.5. Such internal acidification could have been established by Mg^{2+} -dependent ATPase activity *in vivo* before isolation of the granule and maintained by internal buffers such as P_i and proteins (Hutton *et al.*, 1980). A Donnan equilibrium related to insulin and no other acidic proteins in the granule interior could similarly explain these findings. Possibly both mechanisms were operative, since the resting ΔpH could be reduced, but only partially, by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 2) or the combination of valinomycin, nigericin and K^+ (Table 5). The resting ΔpH was also affected by the ionic composition of the medium (Table 3) suggestive of a Donnan equilibrium phenomenon.

Fig. 1. Effect of medium pH on the ΔpH and $\Delta\Psi$ across granule membranes

Granules were incubated either with (○) or without (●) ATP as described in the Experimental section in a buffer containing 25 mM-4-morpholine-ethanesulphonic acid adjusted to the designated pH with Tris. Each plotted value is the mean $\pm$ S.E.M. of the ratio of the intragranular to the medium concentration of the radioisotopic tracer indicated on the left-hand ordinate axis. The right-hand ordinate axis shows the corresponding ΔpH or $\Delta\Psi$ value. Each point is the mean of six to 14 separate incubations performed with three different granule preparations.

Estimation of the intragranular pH *in vivo* must take into account several factors, including, as illustrated in the present study, the cytosolic pH, the presence of ATP and the concentration of permeant anions and bases. The intracellular pH of isolated pancreatic islets determined from their uptake of 5,5-dimethylloxazolidin-2,4-one is in the range 7–7.2 (Hellman *et al.*, 1972; Sener *et al.*, 1978). Such bulk-phase measurements are unlikely to indicate the true cytosolic pH, which may, in any case, be

subjected to localized variation as revealed by microelectrode determinations in other cells (for review, see Roos & Boron, 1981). Nevertheless, if these values are assumed, and given that the $\Delta\mu\text{H}^+$ across granule membrane in the presence of ATP (about 120 mV) were expressed entirely as the ΔpH (about 2 units) the minimum intragranular pH would be 5.0 to 5.2. If the internal pH of freshly isolated granules is assumed as a maximal estimate then the physiological range may be from 5 to 6.

Relationship of pH to insulin storage

The calculated internal pH of the insulin secretory granule approximates the isoelectric point of insulin and corresponds to the pH region of the lowest solubility of the protein either as the free form or the Zn^{2+} -insulin complex (Fredericq & Neurath, 1950; Tanford & Epstein, 1954b). Insulin precipitates, however, may be produced over a broad pH range (pH 4–8 at 5 mg/ml; Tanford & Epstein, 1954b); thus the regulation of the internal pH may not be critical to this activity. The intragranular insulin concentration calculated on the basis of data in the previous publication (Hutton & Peshavaria, 1982) using an intragranular water space of 2 $\mu\text{l}/\text{mg}$ of protein (Table 1) was 200 mg/ml of intragranular water.

The intrinsic dissociation constant of the histidine residues in insulin responsible for its high-affinity binding of Zn^{2+} have been estimated as 6.0 or 6.4 (Tanford & Epstein, 1954a). The apparent dissociation constant in the presence of Zn^{2+} , however, will be considerably lower than this, since Zn^{2+} binds only to the neutral form of the amino acid. It follows that the degree of Zn^{2+} binding and hence the formation of hexameric insulin complexes may be affected by the pH in the range of 4 to 6. The granules presently used contain Zn^{2+} in an approx. 1:1 stoichiometry with insulin (Hutton *et al.*, 1980).

The conversion of amorphous precipitates of Zn^{2+} -insulin to crystalline forms may also be facilitated in the pH range from 5 to 6. From titration data it has been deduced that optimal conditions occur close to the isoelectric point of the molecule (Tanford & Epstein, 1954b).

Regulation of the intragranular pH may be of importance in the proteolytic processing of insulin precursors by the granules, either by providing an optimal pH for activity or in terminating the process. Anglerfish islet secretory granules, for example, contain a thiol proteinase with a pH optimum between 4.5 and 5.5 capable of converting pro-insulin and proglucagon into their respective hormones (Fletcher *et al.*, 1981).

It would seem from each of these considerations that regulation of the intragranular pH may have an important function in the formation and storage of insulin *in vivo*. Conversely it may be argued that the

physical properties of the hormone and processing enzymes have evolved to meet the requirements of storage in an acidic environment.

Relationship to other granule functions

The energy stored in electrochemical gradients across the granule membrane could be used to concentrate solutes in the granule interior. In the case of Ca^{2+} , which is present in high concentrations in granules (Herman *et al.*, 1973) and can be accumulated by an energy-dependent mechanism (Kohnert *et al.*, 1979), a simple relationship to the proton-translocating system is unlikely because of the unfavourable orientation of the $\Delta\Psi$ to inward Ca^{2+} movement. A role in the accumulation of 5-hydroxytryptamine and dopamine (3,4-dihydroxyphenethylamine) by insulin granules (Ekholm *et al.*, 1971) analogous to that operative in the chromaffin granule of the adrenal medulla can be envisaged as discussed previously (Hutton & Peshavaria, 1982).

The chemiosmotic properties of insulin granules revealed in the present experiments were remarkably similar to those of chromaffin granules (Casey *et al.*, 1977; Njus & Radda, 1978; Holz, 1978; Johnson *et al.*, 1978; Njus *et al.*, 1981), amine-storage granules of platelets (Johnson *et al.*, 1978; Wilkins & Salganicoff, 1981) and neurohypophyseal granules (Russell & Holz, 1981). This was evident with respect to the orientation and magnitude of the resting ΔpH and ATP-induced changes in the ΔpH and $\Delta\Psi$ across these granule membranes. This suggested the possibility of a more general role for chemiosmotic phenomena in granule biogenesis or the exocytotic process.

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