Proton Translocation by the Bovine Chromaffin-Granule Membrane

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Bovine chromaffin granules were lysed and their membranes resealed to give osmotically sensitive 'ghosts'. These swell in the presence of salts and MgATP. It is shown that this is due to proton entry accompanied by anions. The rate of swelling depends on the anion present, but swelling is not limited to media containing permeant anions. It is quite marked in solutions of sulphates, phosphates and acetates. It is not uncoupler-sensitive, suggesting that at least one component of swelling is due to coupled proton and anion entry (non-electrogenic proton translocation). Direct measurements of transmembrane pH and potential gradients generated in the presence of MgATP shows that these are rapidly established in sucrose media, and are rather little affected by the presence of salts. They contribute roughly equally to the total protonmotive force. The potential gradient is established very rapidly, but the pH gradient is generated over several minutes. The gradients are not completely dissipated by uncoupler, and it is shown that, in media containing sulphate but no permeant anion, sulphate can be taken up by the 'ghosts'. There thus appear to be two mechanisms of proton translocation across the membrane, both dependent on ATP hydrolysis: an electrogenic transfer of protons, and proton movement linked to an anion transporter of broad specificity.

Chromaffin granules are the site of catecholamine storage in the adrenal medulla. They contain either adrenaline or noradrenaline at a concentration of about 0.7M, together with high concentrations of adenine nucleotides and bivalent cations, and about 200mg of protein/ml (Winkler, 1976; Phillips *et al.*, 1977). Although the concentrations of free unbound components within the granules are unknown, it is clear that the granule membranes are rather permeable to catecholamines at 37°C, and that high concentrations are maintained within the granules by active transport and, presumably, by interacting with internal sites (Sharp & Richards, 1977). ATP-dependent catecholamine uptake is a well-known property of intact isolated granules (Kirshner, 1962; Carlsson *et al.*, 1963).

In a recent series of papers, Radda and his colleagues have proposed a specific mechanism for catecholamine transport in which ATP hydrolysis is coupled to proton movements across the membrane (Bashford *et al.*, 1976; Casey *et al.*, 1976, 1977). This electrogenic pump establishes a pH gradient across the membrane (inside acid) along which catecholamines (weak bases) move, to be bound in a storage complex in the granule matrix. In support of such a model, they and others (Johnson & Scarpa, 1976a,b)

have shown that intact granules are maintained with an internal pH of about 5.5, which decreases on incubation with MgATP unless an uncoupler is added.

To obtain definitive evidence about proton translocation across the chromaffin-granule membrane, we have preferred to work not with intact granules, but with resealed chromaffin-granule 'ghosts'. The intact granules are extremely difficult to purify from mitochondria, and leak ATP and catecholamines during experiments, in addition to undergoing unknown extents of lysis. Their membranes, however, reseal after lysis to form osmotically sensitive 'ghosts' (Phillips, 1977), which can accumulate catecholamines against large concentration gradients (Phillips, 1974a,b). In the present study we used 'ghost' preparations to investigate proton translocation across the membrane; in the following paper the role of proton translocation in catecholamine transport is assessed (Phillips, 1978).

Experimental

Materials

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Atractyloside was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. All other chemicals were obtained as described previously (Phillips, 1977).

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; ATPase, adenosine triphosphatase; Δp , protonmotive force; $\Delta\psi$, membrane potential; $\Delta p\text{H}$, pH gradient.

'Buffered sucrose' contained 10mM-Hepes, pH 7.0, unless otherwise indicated. The pH of 1M-Hepes was adjusted with 4M-NaOH.

Methods

Preparation of chromaffin-granule 'ghosts'. Resealed 'ghosts' were prepared from bovine chromaffin granules by the method described previously (Phillips, 1974a, 1977). This involves lysis of crude granules on a column of Sephadex G-50 equilibrated with 10mM-Hepes, pH 7.0, and their purification in buffered 0.3M-sucrose.

Water-space measurements. Resealed 'ghosts' (about 0.5mg of protein) were suspended in 2ml of buffered 0.3M-sucrose containing $^3\text{H}_2\text{O}$ (0.5 $\mu\text{Ci/ml}$) and inulin- ^{14}C carboxylic acid (100 $\mu\text{g/ml}$, 0.1 $\mu\text{Ci/ml}$). Alternatively, duplicate portions were suspended in media containing $^3\text{H}_2\text{O}$ and ^3H inulin (4 $\mu\text{g/ml}$, 0.5 $\mu\text{Ci/ml}$). Between four and ten replicates were made of each measurement. The 'ghosts' were collected by centrifugation for 20min at 4°C and 40000g; 0.5ml portions of the supernatants were kept for radioactivity counting. The pellets were suspended in 0.5ml of 0.05% Triton X-100 in buffered 0.3M-sucrose containing 5mM-EDTA, and were counted for radioactivity after being shaken well with 2ml of a mixture (1:2, v/v) of Triton X-100 and scintillation fluid [5g of 2,5-diphenyloxazole/litre and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre in toluene]. The supernatants were counted for radioactivity in a similar way. The water space is given by the difference between the volumes of $^3\text{H}_2\text{O}$ and inulin calculated for the pellet.

Measurement of ΔpH , $\Delta\psi$ and Δp . ^{14}C Methylamine and K^{14}CNS were used as tracers for measuring ΔpH and $\Delta\psi$ respectively, external volumes in the 'ghost' pellets being measured with ^3H inulin, as above. The method has been described previously (see, for example, Nicholls, 1974; Johnson & Scarpa, 1976b). The protonmotive force, Δp (Mitchell & Moyle, 1969), is given (in mV) by:

$$\Delta p = \Delta\psi + 60 \Delta\text{pH}$$

where $\Delta\psi$ is the membrane potential (in mV) and ΔpH is the pH gradient across the membrane in pH units. Here we are defining positive values of these quantities as referring to the space inside the 'ghosts' being positive and acidic with reference to the medium. The potential difference was obtained from the relationship:

$$\Delta\psi = 60 \log \left(\frac{[\text{CNS}^-]_i}{[\text{CNS}^-]_o} \right)$$

where $[\text{CNS}^-]_i$ and $[\text{CNS}^-]_o$ refer to the concentrations of thiocyanate inside and outside the 'ghosts' respectively. The weak base methylamine

was assumed to distribute across the membrane in response to the pH gradient so that:

$$\Delta\text{pH} = \log \left(\frac{[\text{B}]_i}{[\text{B}]_o} \right)$$

where $[\text{B}]_i$ and $[\text{B}]_o$ refer to its concentrations inside and outside the 'ghosts' respectively.

'Ghosts' were treated as described for the water-space measurements. The medium was buffered with 30mM-Hepes at pH 7.1 or with 10mM-Hepes at pH 6.3. In the Tables, different experiments refer to different preparations of 'ghosts'. ^{14}C Methylamine hydrochloride was used at a concentration of 15–20 μM (approx. 0.1 $\mu\text{Ci/ml}$); K^{14}CNS was used at a concentration of 25–30 μM (approx. 0.1 $\mu\text{Ci/ml}$). In Expts. 1 and 2 of Table 1 non-radioactive KCNS was included in samples used for ΔpH measurement, and vice versa. Otherwise this was not done. In some experiments, 0.5mM-choline chloride was included, as indicated in the Tables.

From the radioactivities found in the supernatants and pellets, effective methylamine and thiocyanate 'spaces' were calculated for the 'ghosts'. These 'spaces' (in $\mu\text{l/mg}$ of protein) were divided by the water space of the 'ghosts' [taken as 4 $\mu\text{l/mg}$ of protein (see the Results section); no correction was made for changes in this value caused by swelling] to derive the concentration ratios of the solutes between matrix and medium.

Incubations were performed for 5min at 37°C, and then the 'ghosts' were cooled in ice and collected by centrifugation as described above. In all cases control measurements were made on 'ghosts' that were kept in ice. Control values for methylamine were subtracted from experimental values for experiments performed at pH 6.3, since at this pH methylamine should be essentially completely excluded from the interior of the 'ghosts' in the absence of incubation.

Swelling experiments. 'Ghosts' were added to 2ml of 0.3M-sucrose buffered with 50mM-Hepes, pH 7.1, at room temperature (22°C). Turbidity changes were followed at 540nm as described previously (Phillips, 1977). All traces have been corrected for volume changes on the addition of reagents to the cuvettes. Swelling was initiated by adding 50 μl of 0.1M-ATP containing 0.1M- MgSO_4 (pH 7.0).

Uptake of sulphate. 'Ghosts' were incubated at 37°C in buffered 0.3M-sucrose containing the reagents specified in Table 3. Samples (0.1ml) were removed at frequent intervals, and cooled to ice temperature. They were washed through cellulose nitrate filters (0.45 μm pore size; Sartorius, Göttingen, W. Germany) with 6ml of ice-cold buffered 0.3M-sucrose, or, for samples subjected to osmotic shock, with 2ml of 10mM-Hepes, pH 7.0, followed by 4ml of buffered 0.3M-sucrose. Filters were dried and counted for radioactivity in scintillation fluid. In all

experiments time courses of uptake over 15 min were followed.

Protein. This was assayed by the dye-binding method of Bradford (1976).

Results

Exchangeable water space of chromaffin-granule 'ghosts'

The $^3\text{H}_2\text{O}$ -exchangeable water space of intact chromaffin granules is $4.3 \pm 1.1 \mu\text{l}/\text{mg}$ of protein when the granules are suspended in 0.3 M-sucrose containing 10 mM-Hepes (Phillips *et al.*, 1977). Since about 80% of the protein of chromaffin granules is released by osmotic shock (Winkler, 1976), a population of resealed spherical granule membranes might be expected to have a water space of about $20 \mu\text{l}/\text{mg}$ of protein. In practice, we found it difficult to measure this, because of the large quantity of 'ghosts' that is required to obtain a water space that is significantly greater than the volume of water in the pellet that is excluded from the 'ghosts'.

The water space found ($3.65 \pm 0.54 \mu\text{l}/\text{mg}$ of protein, mean $\pm$ S.D. for determinations on four different 'ghost' preparations) is comparatively small. The reason for this is likely to be that most of the granules, after lysing as the osmotic pressure is decreased, reseal before the osmotic pressure of the medium is restored by addition of sucrose to 0.3 M. On addition of sucrose they therefore shrink so that the osmotic pressure inside them remains constant. The lysis medium contains 10 mM-Hepes, an impermeant zwitterionic buffer (Good *et al.*, 1966), and it is therefore likely that the resealed 'ghosts' contain this at a fairly high concentration, as well as some residual sucrose. Indeed, electron micrographs of resealed 'ghosts' (Phillips, 1974a) suggest that they are mostly not hollow spheres but are collapsed spheres of a variety of shapes, although few unsealed structures are present.

To determine the exact concentration of solutes within the resealed 'ghosts', it is necessary to know the composition of the medium at the time of re-sealing. Granules suspended in 0.3 M-sucrose, buffered with 10 mM-Hepes, are passed down a column of Sephadex equilibrated with the same buffer, but in the absence of sucrose. As sucrose is removed from the medium, the granules lyse and their soluble contents are also removed by the gel-filtration process. If the 'ghosts' reseal while still on the column they will contain 10 mM-Hepes and a concentration of sucrose that will depend on how much has been removed by the column. After washing through the column, the 'ghost' suspension medium is adjusted to a concentration of 0.3 M-sucrose; any resealed 'ghosts' will shrink by water loss until their internal osmotic pressure equals that of the medium. 'Ghosts'

that reseal after sucrose addition will contain 0.3 M-sucrose and 10 mM-Hepes.

We do not know at what point in this procedure resealing occurs. The low water space found suggests that shrinking occurs after resealing; this process will lead to a concentration of Hepes within the 'ghosts'. If this has occurred, the Hepes will be concentrated $[(4.3 \times 5) \div 3.65] = 6$ -fold, to about 60 mM (containing 16 mM- Na^+). However, we cannot be certain about this, since we do not know that a 'ghost' resealing in 0.3 M-sucrose will form a sphere and not a collapsed structure of smaller volume.

The large standard deviation of the results obtained for the water space reflects the difficulty of making the measurements. We have used a value of $4 \mu\text{l}/\text{mg}$ of protein in all calculations in this paper in order to err on the side of underestimating concentrations of components within 'ghosts'.

Swelling of 'ghosts' induced by MgATP

Methods for studying the ionic permeability of chromaffin-granule membranes by conducting swelling experiments with 'ghosts' have been described previously (Phillips, 1977). The same approach was used to study ion permeability in the presence of ATP: 'ghosts' suspended in 0.3 M-sucrose containing 50 mM-Hepes at pH 7.1 were subjected to shrinking by addition of a salt solution to a concentration of 50 mM. It was found that swelling could be initiated, not only by the addition of ionophores as in the previous work (Phillips, 1977), but also by the addition of MgATP. Swelling does not occur in the absence of salt, as discussed below.

Some results are illustrated in Fig. 1 for the addition of 2.5 mM-MgATP (2.5 mM- Na_3ATP containing 2.5 mM- MgSO_4) to 'ghosts' in the presence of 50 mM-KI. Similar results were found with many other potassium salt solutions.

The swelling has the following characteristics.

(1) The trace falls rapidly to the original baseline of the 'ghost' suspension, reaching a plateau value within about 3 min (the traces in the Figures have been corrected for volume changes on adding reagents to the cuvettes). (2) The swelling rate is increased by addition of nigericin. In this case the absorbance falls far below its starting value, and continues to decrease for many minutes. Nigericin has a similar effect if added when the absorbance has reached a plateau value with MgATP. (3) The swelling rate is unaffected by addition of uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), and slightly decreased by addition of valinomycin (Fig. 2). In combination, valinomycin and the uncoupler are rather less effective than nigericin in stimulating swelling. (4) As described below, the rate of swelling is markedly dependent on the anion present, but is independent of the cation,

Fig. 1. Swelling of chromaffin-granule 'ghosts' induced by MgATP

For this, 50 mM-KI was added to 'ghosts' suspended in buffered 0.3 M-sucrose. At the times indicated 2.5 mM-MgATP (A) and 35 nM-nigericin (N) were added. Absorbance changes were followed at 540 nm.

Fig. 2. Swelling of chromaffin-granule 'ghosts' induced by MgATP

For this, 50 mM-KI was added to 'ghosts' (same preparation as in Fig. 1) suspended in buffered 0.3 M-sucrose. Additions were: 2.5 mM-MgATP (A); 0.5 μ M-valinomycin (V); 5 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (F). Absorbance changes were followed at 540 nm.

These observations suggest that, in the presence of MgATP, I^- ions are being transferred to the interior of the 'ghosts', accompanied by protons. The lack of effect of cations on the rate, and the fact that the rate is higher with MgATP than with valinomycin (Fig. 2), suggest that protons are the

counterions to I^- rather than K^+ ions. In this context, proton entry cannot be distinguished from OH^- exit.

A variety of mechanisms could account for such movements of ions. The two that seem most probable are illustrated in Scheme 1. In Scheme 1(a) proton entry is shown as a non-electrogenic process, closely linked to anion entry. In Scheme 1(b) proton entry is shown as electrogenic, I^- ions diffusing passively across the membrane, following their electrochemical gradient. Both processes would be stimulated by nigericin, which removes the gradient of protons, as K^+ ions move into the 'ghosts' down their concentration gradient.

Swelling caused by electrogenic proton entry would be expected to be inhibited by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, owing to leakage of protons across the membrane. In addition, valinomycin, which leads to the establishment of a potassium diffusion potential (positive inside), would also be inhibitory. Neither reagent is markedly inhibitory (Fig. 2), suggesting that at least some component of the swelling process must be due to a non-electrogenic mechanism.

The absorbance changes cannot be interpreted in detail, since they depend on factors such as the refractive index of the medium as well as the shape and size of the 'ghosts'. However, they clearly fall below the baselines for the initial 'ghost' suspensions, and further than is observed when 'ghosts' in salt solutions are swollen by the addition of ionophores (Phillips, 1977); for comparison, swelling of 'ghosts' in 50 mM-KCl containing trimethyltin and nigericin is shown in Fig. 3 (bottom trace). This suggests that the osmotically active ions are reaching greater concentrations inside the 'ghosts' than in the supporting medium; swelling of the 'ghosts' (water entry) keeps the internal osmotic pressure constant. Equilibrium is presumably reached when the rate of passive diffusion out of the 'ghosts' (leakage) equals the rate of entry.

Reversal of 'ghost' swelling by addition of ionophores

If K^+ and I^- are being transported inside the 'ghosts' to concentrations above those outside in the supporting medium (leading to swelling beyond the baseline of the traces), it should be possible to reverse the swelling by adding ionophores that permit the ions to leak out down their concentration gradients. We tested this by a series of experiments in which KI was replaced by 50 mM-KCl, since we were able to use trimethyltin as an ionophore for Cl^- . It is known to catalyse anion/hydroxyl ion exchange across membranes (Selwyn *et al.*, 1970); 'ghosts' in the presence of KCl and nigericin swell when it is present in the medium (Fig. 3, bottom trace) because nigericin permits K^+ entry (down its concentration

Scheme 1. *Schemes for non-electrogenic and electrogenic models of proton translocation*

MgATP is shown as a substrate for a proton-translocating ATPase in the membrane of a chromaffin-granule 'ghost'. In (a), proton movement is coupled to movement of the anion, X^- (this cannot be distinguished experimentally from an inward movement of X^- coupled to outward movement of OH^-). The proton gradient established can be dissipated by H^+/K^+ exchange in the presence of nigericin (NIG). In (b) proton translocation is electrogenic: proton entry is accompanied by passive movement of X^- across the membrane. Such a process is expected to be inhibited in the presence of either an uncoupler (UNC) or valinomycin (VAL); the latter leads to the formation of a membrane potential (positive inside) in the presence of potassium salts.

gradient) and the resulting pH gradient can be dissipated by trimethyltin-catalysed Cl^-/OH^- exchange.

'Ghosts' shrunk in the presence of 50mM-KCl swell rapidly when MgATP is added (Fig. 3, upper trace), and this rate is scarcely affected by adding trimethyltin at the same time. Nigericin, by contrast, increases the swelling rate (Fig. 3, middle trace), as is the case in the presence of KI, and also greatly increases its extent. The nigericin-accelerated swelling is little affected initially by adding trimethyltin (Fig. 3, bottom trace), but if this ionophore is added after much longer times of incubation, some reversal of the absorbance change occurs; this is in contrast with the decrease in absorbance found when trimethyltin is added to 'ghosts' with nigericin but in the absence of ATP.

We interpret this as being evidence for the existence of K^+ and Cl^- inside the 'ghosts' at a concentration above that in the medium. Since nigericin is already present, the addition of trimethyltin permits movement of both ions out of the 'ghosts', down their concentration gradients; subsequent water movement leads to shrinking. From the known mechanism of nigericin, this is evidence for MgATP-catalysed H^+ and Cl^- movements across the membrane; the experiment does not discriminate between passive Cl^- entry through the membrane in response to electro-

genic proton movement, and coupled proton and anion movements.

Trimethyltin-induced reversal could not be demonstrated if the external KCl concentration was increased to 150mM. Addition of the ionophore always led to a slight decrease in absorbance due to entry of ions into the 'ghosts'.

A similar experiment was performed in a medium containing 50mM-KI and nigericin. Addition of valinomycin after about 20min of incubation with MgATP leads to a slight reversal of the decrease in absorbance as K^+ leaves the 'ghosts' accompanied in this case by a passive exit of permeant I^- .

Swelling of 'ghosts' in the presence of salts

In initial experiments, swelling of 'ghosts' was initiated by addition of 2.5mM-ATP (sodium salt) containing 1.25mM- $MgSO_4$, since excess ATP is required for catecholamine incorporation (Phillips, 1974b). However, it was found that under these conditions a slow swelling of the 'ghosts' occurred in the basic medium (0.3M-sucrose, 50mM-Hepes) in the absence of added salt. To avoid this, $MgSO_4$ was added at the same concentration as ATP, when no swelling occurs, although there is an immediate rise in absorbance, presumably due to 'ghost' aggregation in the presence of Mg^{2+} ions (Fig. 4a). The two

Fig. 3. Effect of ionophores on swelling of chromaffin-granule 'ghosts'

For this, 50 mM-KCl was added to 'ghosts' suspended in buffered sucrose. Subsequent additions were 2.5 mM-MgATP (A), 0.5 mM-trimethyltin chloride (T) and 35 nM-nigericin (N). The upper two traces are composites from three and two different experiments respectively. Absorbance changes were followed at 540 nm.

Mg²⁺ concentrations gave almost identical results when salts were added to the suspension medium.

MgATP stimulated swelling in the presence of many salts. Results for some potassium salts are shown in Fig. 4(a). Unlike the control trace, there is no increase in absorbance (aggregation may be accompanied by an initial rapid phase of swelling on ATP addition), but there is relatively slow swelling over several minutes. The order of effectiveness of anions at pH 7.1 was:

It should be noted that several of these anions (e.g. SO_4^{2-} and HPO_4^{2-}) have very low passive permeability through the membrane (Phillips, 1977).

As discussed above for KI, both valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone had little effect on the observed swelling rates. Nigericin, on the other hand, stimulated swelling in the presence of permeant anions (Fig. 4b); in this case, the stimulation of swelling rate was related to the anion's passive permeability, being greatest for thiocyanate, the most permeant anion tested. 'Ghosts' do not swell under these conditions in the absence of MgATP.

The effect of different cations was investigated, by using both chlorides and sulphates (in the absence of ionophores): 2.5 mM-MgATP induced swelling in the presence of 50 mM-NaCl and -choline chloride at rates only slightly less than that with KCl; swelling was much faster in NH_4Cl , and was equal to the rate

Fig. 4. Swelling of chromaffin-granule 'ghosts' induced by MgATP in the presence of potassium salts

For this, 50 mM-potassium salts were added to 'ghosts' suspended in buffered sucrose. In (a) 2.5 mM-MgATP was added as shown. In (b) 35 nM-nigericin was added after the potassium salt solution; 2.5 mM-MgATP was added 1–2 min later. (a) and (b) are taken from experiments on the same preparation of 'ghosts'. Absorbance changes were followed at 540 nm.

when nigericin was added to KCl. Swelling in Na_2SO_4 and $(\text{NH}_4)_2\text{SO}_4$ was very slightly slower than swelling in K_2SO_4 . It should be stressed, however, that the cation seemed to have very little effect on the rate. Bivalent cations lead to marked aggregation of the 'ghosts', and were not investigated.

The swelling rate was markedly dependent on the concentration of MgATP, showing saturation kinetics, with half-saturation occurring at a concentration of about 0.5 mM. The following did not support swelling, and were inhibitory to the action of MgATP (Fig. 5): ADP and MgADP, adenylyl imidodiphosphate and adenylyl [β -methylene]diphosphonate (both in the presence of MgSO_4). Three inhibitors of the chromaffin-granule ATPase, dicyclohexylcarbodi-imide (Bashford *et al.*, 1976), trimethyltin and quercetin (D. K. Apps, personal communication) were slightly inhibitory in the presence of KI (Fig. 5b). The traces were not affected by the addition of reserpine, a potent inhibitor of catecholamine transport by the 'ghosts'.

Fig. 5. Effect of inhibitors on swelling of chromaffin-granule 'ghosts'

Inhibitors were added to 'ghosts' suspended in buffered sucrose containing 50 mM-KI. (a) At the beginning of the trace the following additions were made: (i) 2.5 mM-adenylyl imidodiphosphate containing 2.5 mM-MgSO₄; (ii) 2.5 mM-adenylyl [β -methylene]diphosphonate containing 2.5 mM-MgSO₄; (iii) 2.5 mM-MgATP (control); 2.5 mM-MgATP was added to the first two traces at the times indicated (ATP). In (b) 2.5 mM-MgATP was added at the beginning of each trace. The following additions had previously been made to the 'ghosts': (i) 2.5 mM-MgADP; a further addition of 2.5 mM-MgADP was made at the time indicated by the arrow; (ii) 'ghosts' (0.44 mg of protein/ml) had been treated with dicyclohexylcarbodi-imide (0.5 mM) for 40 min at 22°C before the experiment; (iii) 0.5 mM-trimethyltin chloride; (iv) quercetin (20 μ g/ml); (v) no addition (control). Absorbance changes were followed at 540 nm.

Direct measurement of transmembrane pH and potential gradients

Direct measurements of pH and potential gradients across the membrane were made by collecting 'ghosts' by centrifugation from solutions containing [^{14}C]methylamine or K^{14}CNS in the presence of [^3H]inulin as a marker for extraparticulate water in the pellets. These experiments require large quantities of 'ghosts' in order to obtain pellets with included volumes significantly higher than excluded volumes, and this limits the amount of detailed information that can be obtained.

The weak base methylamine is found in the pellet distributed in accord with the pH values of the two compartments; this technique is useful as long as the internal pH is lower than the external pH. It could not be used to measure very small pH differences, as some methylamine binds to the membranes [this result is in contrast with that obtained by Johnson & Scarpa (1976a), who found no binding to intact chromaffin

granules]. The binding was minimized by inclusion of 0.5 mM-choline chloride or of 40 mM-salt solutions in the incubation media, but even so some methylamine 'space' (methylamine included in 'ghosts' or bound to their membranes) was found in 'ghosts' in the presence of MgATP at ice temperatures. It was difficult to correct for this, however, as lower spaces were sometimes found in incubations with uncouplers present, suggesting that the values at 0°C were partly due to a real small pH difference across the membrane in the absence of incubation. This is quite plausible, since the exact composition of the solution inside the 'ghosts' is not known. Because of the logarithmic relationship between methylamine space in the pellet and the calculated ΔpH , there is a large error in ΔpH at low values, and measurements of small gradients must be treated with caution. Because the [^{14}C]methylamine background binding value is not known accurately, no blanks have been subtracted from the data presented in Table 1. The smallest pH gradient measured, however, was 0.20 pH unit, which suggests that the error arising from ignoring methylamine binding is probably 0.2 pH unit or less. All measurements of pH gradients were made in duplicate, as described under 'Methods'. Mean values are given in the Tables, since duplicates agreed closely. The large amount of purified material required prevented more extensive measurements.

Potential gradients across the membrane were monitored by using the distribution of the lipophilic thiocyanate ion. This distributes according to the Nernst equation when the 'ghosts' have a potential difference across their membranes with inside positive. In the absence of MgATP at 0°C, and in the absence of a permeant cation, the thiocyanate space of 'ghost' pellets was found to be the same as the inulin space, i.e. all thiocyanate is excluded from the interior of the 'ghosts', and none is bound to the membranes. A small and variable space is found when MgATP is added at this temperature. Its size correlated with the space found on incubation at 37°C, and it was therefore ignored in calculations of $\Delta\psi$. However, as with ΔpH measurements, it introduces an element of uncertainty for small values of $\Delta\psi$ (below 20 mV).

Values of potential difference ($\Delta\psi$), pH gradient (ΔpH) and protonmotive force (Δp) are presented in Table 1. 'Ghosts' were incubated in the basic sucrose medium, buffered at pH 7.1, with the supplements shown. Catecholamine uptake is optimal at this pH, and the inhibitor reserpine was included when the effects of different salts were examined (Expt. 2), as the 'ghost' preparations contain low concentrations of amines.

Substantial pH gradients were established, both with the basic sucrose medium (low ionic strength) and in the presence of salts. ΔpH was greatest in the presence of permeant ions such as I^- , but was

Table 1. Measurements of $\Delta\psi$, ΔpH and Δp across the chromaffin-granule 'ghost' membrane

'Ghosts' were incubated for 5 min at 37°C with 6 mM-ATP and 3 mM-MgSO₄ at an external pH of 7.1. The sucrose medium was supplemented with salts at a concentration of 40 mM where indicated. In Expts. 2 and 3, $\Delta\psi$ and ΔpH were measured on different preparations of 'ghosts'; all samples in Expt. 2 contained 5 μ M-reserpine. The samples in Expt. 1 contained 0.5 mM-choline chloride. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide.

Expt. No.	Medium	Additions	$\Delta\psi$ (mV)	ΔpH (pH units)	Δp (mV)
1	Sucrose	None	74	1.26	150
		10 μ M-FCCP	26	1.01	87
2	Na ₂ SO ₄	None	74	1.17	144
		10 μ M-FCCP	28	0.46	56
	NaF	None	67	1.22	140
		10 μ M-FCCP	15	0.74	59
	NaCl	None	32	1.69	133
		10 μ M-FCCP	16	0.64	54
	NaI	None	16	1.88	129
		10 μ M-FCCP	8	0.76	54
3	K ₂ SO ₄	None	53	1.29	130
		10 μ M-FCCP	13	0.29	30
	KCl	70 nM-nigericin	80	0.20	92
		50 μ g of quercetin/ml	36	1.16	106
		None	32	1.53	124
		10 μ M-FCCP	11	0.35	32
		70 nM-nigericin	56	0.56	90

nevertheless substantial in the presence of impermeant ions such as SO₄²⁻ and F⁻; a converse effect was seen on $\Delta\psi$, so that Δp remained essentially constant in the high-ionic-strength media tested. Δp was decreased to about 25–60% of its original value in the presence of uncoupler (10 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide); nigericin, in the presence of 40 mM-potassium salts, greatly decreased ΔpH , but there was a corresponding increase in $\Delta\psi$.

The experiments were all performed with an ATP concentration twice that of MgSO₄, conditions optimal for catecholamine uptake (Phillips, 1974b). The ATPase, however, has optimal activity with equimolar Mg²⁺ and ATP (Muller & Kirshner, 1975), as does swelling. Measurements with equimolar Mg²⁺ and ATP showed essentially unchanged values of $\Delta\psi$ and ΔpH .

The chromaffin-granule ATPase has a broad pH optimum between pH 6 and pH 8, but catecholamine uptake is very much decreased below pH 6.5 (Taagner,

Fig. 6. Establishment of pH gradient and membrane potential by chromaffin-granule 'ghosts' 'Ghosts' were incubated at 37°C as described under 'Methods' with 6mM-ATP containing 3mM-MgSO₄ at pH6.3. In (a) the medium also contained 40mM-NaCl. Samples were removed for assay of ΔpH (a) and for $\Delta\psi$ (b).

Table 2. Measurements of $\Delta\psi$ and ΔpH across the chromaffin-granule 'ghost' membrane

'Ghosts' were incubated as described for Table 1, expect that the pH of the medium was 6.3. A dash indicates that no measurement was made. $\Delta\psi$ and ΔpH were measured on the same preparations of 'ghosts'. All samples except those of Expt. 4 contained 0.5mM-choline chloride. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Expt. No.	Medium	Additions	$\Delta\psi$ (mV)	ΔpH (pH units)
1	NaCl	None	26	0.70
		5 μM -FCCP	23	0.38
2	NaCl	None	20	0.98
		5 μM -Reserpine	20	1.05
	Na ₂ SO ₄	None	60	0.35
		5 μM -Reserpine	58	0.24
3	Na ₂ SO ₄	None	77	0.34
		5 μM -FCCP	50	0.14
4	Na ₂ SO ₄	None	72	—
		5 μM -FCCP	41	—
		50 μg of quercetin/ml	48	—
		0.5mM-Trimethyltin	43	—
		50 μg of atractyloside/ml	—	0.88
		35nM-Nigericin	—	<0
5	Sucrose	None	78	0.89
		5 μM -FCCP	30	0.42
		20 μg of quercetin/ml	—	0.89
		0.5mM-Trimethyltin	—	0.26
		50 μg of atractyloside/ml	—	0.88
		KCl	—	1.34

1972). A series of experiments was therefore performed in which the 'ghosts', prepared as usual at pH 7.0, were transferred to a medium at pH 6.3, generating a pH gradient across the membrane (inside alkaline). Marked pH gradients were generated extremely rapidly in the presence of ATP, the pH being reversed within about 1 min at 37°C (Fig. 6), to give internal pH values well below the pH of the external medium. Some results are presented in Table 2.

As at pH 7.1, the greatest pH gradients are generated when there is a high concentration of permeant anion present. With the basic sucrose medium, and when this is supplemented with 40mM-Na₂SO₄, a greater potential difference is developed. $\Delta\psi$ values are very similar to those found at pH 7.1. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone fails to eliminate the pH and potential gradients, although these are decreased; in other words, even in the presence of the uncoupler, the internal pH decreases below that of the medium.

Quercetin (20 $\mu\text{g}/\text{ml}$) has rather little effect,

consistent with its marginal effect in swelling experiments. Trimethyltin (in the absence of Cl⁻) is similar to quercetin. Nigericin completely eliminated the pH gradient in the presence of external K⁺; [¹⁴C]-methylamine was completely excluded from the 'ghosts' in this case. Atractyloside and reserpine were essentially without effect.

The time course of generation of the gradients at pH 6.3 is shown in Fig. 6. A NaCl medium was used for ΔpH (Fig. 6a) in view of the large values obtained. The method is not very accurate for kinetic measurements of this sort, since the 'ghost' preparations are cooled in ice after their incubation, and are then centrifuged for 20min at 4°C before removal of the incubation medium. The initial ΔpH is given as -0.8; this is assumed, since the 'ghosts' were prepared in Hepes, which, at a concentration of 50mM (probable value for the 'ghost' interior) had a pH of 7.1. Nevertheless, the time courses of ΔpH and $\Delta\psi$ generation are rather different; the potential difference is established immediately, and is maintained

Table 3. *Uptake of $^{35}\text{SO}_4^{2-}$ by chromaffin-granule 'ghosts'*

Each experiment refers to a different preparation of 'ghosts', incubated as described under 'Methods' in the presence of 4.5 mM-ATP, and Mg^{2+} , SO_4^{2-} and 5-hydroxytryptamine at the concentrations shown. Radioactive sulphate was present at approx. 1 $\mu\text{Ci/ml}$.

Expt. No.	$[\text{Mg}^{2+}]$ (mM)	$[\text{SO}_4^{2-}]$ (mM)	[5-Hydroxytryptamine] (μM)	[Protein] (mg/ml)	SO_4^{2-} incorporated (nmol/5 min per mg of protein)
1	0.20	0.21	0	0.11	1.4
	0.20	0.23	16	0.11	2.4
2	0.27	0.32	0	0.19	0.6
	0.27	0.36	45	0.19	2.0
3	0.50	0.53	0	0.15	2.9
	0.70	0.82	71	0.08	3.9
4	0.85	0.98	85	0.20	4.8

essentially unchanged over 15 min while the pH gradient builds up steadily to a plateau value. It resembles the time course of MgATP-induced swelling in 50 mM-KCl (Fig. 3).

Counter-ion for proton translocation

A striking feature of Table 2 is the magnitude of the pH gradients observed, even in the presence of supposedly impermeant anions such as SO_4^{2-} and F^- . Although potential differences are greater with such anions than with permeant anions, it is inconceivable that the large proton movements revealed by the ΔpH measurements could be completely electrogenic; in other words, anion uptake must accompany these proton movements.

The most likely anion seemed to be SO_4^{2-} , since MgSO_4 is used in all the incubation media; the concentrations of Cl^- and other anions in the sulphate media of Table 1 are low, being of the order of 50 μM . We therefore attempted to demonstrate uptake of $^{35}\text{SO}_4^{2-}$ by the 'ghosts' in the presence of ATP, but in the absence of other anions, by filtering samples at intervals and comparing their radioactivity with that of 'ghosts' subjected to osmotic shock, as in assays for catecholamine uptake (Phillips, 1974a). To increase the specific radioactivity of the sulphate, concentrations of MgSO_4 that were well below the ATP concentrations were used, producing rather suboptimal conditions for proton translocation. Results of some experiments are presented in Table 3. For simplicity, single time points taken from 15 min time courses are given. SO_4^{2-} appeared to leak from the 'ghosts' after 5 min in some preparations.

The results obtained were rather marginal as the $^{35}\text{SO}_4^{2-}$ incorporated was generally low compared with the background; some preparations of 'ghosts' were nearly inactive, suggesting that the assay method is not satisfactory. However, there did seem to be some incorporation, sensitive to osmotic shock,

that was roughly dependent on the concentration of MgSO_4 present.

Discussion

Interpretation of swelling experiments

Chromaffin-granule 'ghosts' swell in a predictable way when suspended in salt solutions containing appropriate ionophores or permeant ions (Phillips, 1977). Marked swelling also occurs on the addition of MgATP. It is almost certain that hydrolysis of ATP is required for this phenomenon. Thus MgADP and the ATP analogues adenylyl [β -methylene]diphosphate and adenylyl imidodiphosphate fail to support swelling, and also inhibit MgATP-induced swelling; treatment with the ATPase inhibitor dicyclohexylcarbodi-imide (Bashford *et al.*, 1976) is inhibitory.

A possible interpretation of the swelling traces is that MgATP binding or hydrolysis alters the ionic permeability of the membrane, without catalysing active transport of ions. Brierley and his co-workers, for example, have suggested that 'energization' of heart mitochondria alters their cation permeability (Brierley, 1974); the consequence is that cations enter electrogenically with permeabilities in the order $\text{Na}^+ < \text{Tris} < (\text{CH}_3)_4\text{N}^+ < \text{Li}^+ < \text{K}^+$ (Brierley *et al.*, 1971). It seems unlikely that the present results with chromaffin-granule 'ghosts' could be explained in a similar way, for the following reasons. (1) NaCl, KCl and choline chloride have essentially identical effects. (2) Swelling occurs in the presence of apparently impermeant anions, such as SO_4^{2-} and HPO_4^{2-} , the permeabilities of which would also have to be increased. (3) The marked stimulatory effect of nigericin cannot be explained by this type of model. (4) Models invoking solely 'downhill' solute entry cannot account for the apparent accumulation of solute within the 'ghosts' that is suggested by the results shown in Fig. 3.

As discussed above, we therefore interpret the swelling experiments in terms of a non-electrogenic linked proton/anion symport (or hydroxyl ion/anion antiport) across the membrane; in the presence of excess ATP this can catalyse accumulation of anions to a concentration exceeding that in the medium; it has broad specificity: although rates vary considerably with the anion present, of those tested only F^- fails to support swelling as assayed by absorbance change. Swelling by such a mechanism is relatively insensitive to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or valinomycin, but is stimulated by nigericin (in the presence of potassium salts) or by NH_4^+ ions (which assist swelling by dissipating the pH gradient after entry of NH_3 molecules).

Rapid MgATP-induced swelling in solutions containing impermeant anions such as SO_4^{2-} and HPO_4^{2-} is the main observation that excludes interpretations based solely on a model invoking electrogenic proton translocation (although the pH-gradient measurements provide supporting evidence, as discussed below). The fact that swelling is insensitive to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone could in itself be explained if the pH inside the 'ghosts' is slightly higher than that of the medium, owing to the higher internal ionic strength and buffer concentration. Electrogenic proton translocation (generating a potential with inside positive, but failing to generate a pH gradient with inside acid) would then be insensitive to uncouplers, and would cause swelling by passive electrophoretic anion entry. This could account, at least partially, for the high swelling rates seen with permeant anions, since there seems little doubt that, as discussed below, electrogenic proton entry accompanies the non-electrogenic mechanism discussed above and outlined in Scheme 1.

The case of nigericin stimulation of swelling is interesting. In the presence of nigericin and K^+ pH gradients are very much decreased, but the potential gradients obtained (Table 1) are increased. The greatest stimulation of swelling rate is seen in the presence of permeant anions (Fig. 4), which presumably respond to the raised potential by entering the 'ghost' through the membrane, a response that is not available to the impermeant ions such as SO_4^{2-} , the entry of which is directly coupled to proton translocation.

Measurements of pH and potential gradients

Energization by MgATP leads to the generation of pH and potential gradients across the membrane (inside acid and positive). Can these be explained by electrogenic proton translocation?

The gradients are sensitive to uncoupler (10 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), although not abolished by it under our

conditions, suggesting that at least a component of proton entry is electrogenic. However, marked gradients are generated in the presence of SO_4^{2-} ions, both in the basic sucrose medium and in the sulphate-supplemented medium, a result consistent with the swelling observed under similar conditions. We can make a rough calculation of the proton flux required to generate pH gradients of the size found. Titration showed that 50 mM-Hepes, pH 7.18, is adjusted to pH 5.98 ($\Delta pH = 1.2$ pH units) by the addition of 15.0 μmol of HCl/ml. Since the internal water space of the 'ghosts' is 4 μl /mg of protein, a pH gradient of 1.2 pH units (a typical value from Table 1) would thus be generated by an influx of 60 ng-ions of H^+ /mg of protein (to give 15 ng-ions of H^+ / μl) if the internal buffer concentration is 50 mM (30 mM-Hepes would require about two-thirds as much H^+). Removal of these protons has no effect on the pH of the supporting medium, which is buffered with 30 mM-Hepes. If this flux of protons was purely electrogenic (i.e. not accompanied by anion entry or cation efflux) this would generate a membrane potential (dependent on the number of protons translocated, and not on their internal activity, or pH) of the order of 300 mV, as calculated from the Nernst equation, far higher than the potential differences observed. We therefore conclude that the pH gradients measured in Tables 1 and 2 are due, at least in part, to proton movement accompanied by transfer of anions across the membrane.

Although this is readily explicable for a medium containing permeant Cl^- ions, for the SO_4^{2-} medium this result and the swelling experiments suggest the entry of SO_4^{2-} , known to have a very low permeability through the membrane (Phillips, 1977). The experiments with $^{35}SO_4^{2-}$ were an attempt to demonstrate this directly. Extrapolating the results of Table 3 to the conditions of the pH-gradient measurements (3 mM-MgSO₄) suggests an uptake of about 15 ng-ions of SO_4^{2-} /mg of protein in 5 min. This would give an internal concentration of about 4 μg -ions of SO_4^{2-} /ml, at least partially compensating for the proton influx of 15 ng-ions/ml. This uptake could result either from an increased passive SO_4^{2-} permeability in the energized membrane, or from directly linked proton and sulphate movements.

Since swelling occurs in a variety of salt solutions, it seems possible that several anions may accompany proton entry in this unusual way, in other words that there is non-specific anion entry. This does not occur simply in response to an imposed potential difference, since 'ghosts' do not swell when valinomycin is added to suspensions in potassium salts of these acids (Phillips, 1977), but must be linked in some way to proton translocation or ATP hydrolysis.

Does this imply that the chromaffin-granule membrane contains two separate mechanisms for coupling proton translocation to ATP hydrolysis,

one electrogenic, the other linked to anion movement (Scheme 1)? Although this cannot be answered at the moment, the fact that the Δp values in Table 1 do not vary much when the salt present is changed from Na_2SO_4 or NaF to NaI , although the relative contributions of $\Delta\psi$ and ΔpH do change, suggests that a single proton-translocating ATPase is probably present. This conclusion is consistent with investigations of the ATPase activity of the membrane. Studies in which the enzymic activity was solubilized by two different procedures (Apps & Reid, 1977), activity staining after polyacrylamide-gel electrophoresis and studies with inhibitors (D. K. Apps & L. A. Glover, unpublished work) have failed to provide any evidence for more than one enzyme.

Although this recent work is not conclusive, it suggests that the chromaffin-granule membrane contains one ATPase. This functions to couple ATP hydrolysis with proton translocation, the proton movement being linked to anion movement of broad specificity. The role of proton translocation in catecholamine transport is assessed in the following paper (Phillips, 1978); the physiological role of the anion movements is unknown.

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