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A sodium/proton antiporter in chromaffin-granule membranes

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Chromaffin granules, the secretory vesicles of the adrenal medulla, have a Na^+/H^+ exchange activity in their membranes which brings their proton gradient into equilibrium with a Na^+ gradient. This explains why Na^+ is mildly inhibitory to amine transport (which is driven by the H^+ gradient). The activity can be demonstrated by using accumulation of $^{22}Na^+$ in response to a pH gradient that is either imposed by diluting membrane 'ghosts' into alkaline media, or generated by ATP hydrolysis. It can also be monitored indirectly by fluorescence measurements in which the pH inside 'ghosts' is monitored by quenching of a fluorescent weak base. This method has been used to monitor Na^+ entry into acid-loaded 'ghosts' or H^+ entry into Na^+-loaded 'ghosts'. Na^+-jump experiments also lead to acidification of the interior, as demonstrated by methylamine accumulation. The exchanger appears to be reversible and non-electrogenic, with a stoichiometry of 1:1. Using an indirect assay we measured an apparent K_m for Na^+ of 4.7 mM, and a K_i for amiloride, a competitive inhibitor, of 0.26 mM. Direct assays using $^{22}Na^+$ suggested a higher K_m. Ethylisopropylamiloride was not inhibitory.

INTRODUCTION

Chromaffin granules, the secretory vesicles of the adrenal medulla, contain high concentrations of catecholamines and nucleotides as well as various proteins and biologically active peptides. Like many other secretory vesicles, they have an acidic matrix; in isolated granules this appears to be buffered at about pH 5.7 (Johnson & Scarpa, 1976). Similar or slightly lower values are probably found in intact tissues (Holz *et al.*, 1983; Bevington *et al.*, 1984). This acidic milieu is generated and maintained by a V-type (Mellman *et al.*, 1986; Pederson & Carafoli, 1987) proton-translocating ATPase which utilizes cytosolic ATP for inwardly directed electrogenic proton translocation. The resulting proton and potential gradients are used as an energy source for catecholamine transport (see review by Njus *et al.*, 1987*a*).

In this paper we show that chromaffin-granule membranes contain a reversible Na^+/proton antiporter. We suggest that this is of sufficient capacity to equilibrate Na^+ and H^+ across the membrane, so that the granules have a Na^+ gradient as well as a proton gradient that may be used as an energy source. This would be consistent with the long-standing observation that Ca^{2+} transport by the granules is Na^+-dependent, and is not linked directly to protons (Phillips, 1981; Krieger-Brauer & Gratzl, 1982).

MATERIALS AND METHODS

Materials

Bovine adrenal glands were placed on ice within about 30 min of slaughter. They were transported to the laboratory and chromaffin granules were prepared within the next 2 h (Phillips, 1974*a*). All radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was a gift from Dr. R. Kraayenhof (Vrije University, Amsterdam, The Netherlands), and ethylisopropylamiloride was a gift from Professor F. Lang, Institut für Physiologie, University of Innsbruck, Austria. Cellulose nitrate filters (pore size 0.45 μm) were from Schleicher and Schüll, Dassel, West Germany.

Hepes and Mes were generally prepared as 1 M solutions and adjusted to the required pH with NaOH, KOH or tetramethylammonium (TMA) hydroxide. pH values are quoted for the concentration used in experiments. EGTA was also adjusted to the required pH; it was included in most media to decrease Ca^{2+} movements coupled to Na^+.

Methods

Preparation of 'ghosts'. Resealed chromaffin-granule 'ghosts' were prepared by the method of Apps *et al.* (1980): this involves lysis of fresh granules by 50-fold dilution into a lysis buffer, collection by centrifugation, purification through a sucrose/^2H_2O gradient, and storage at −20 °C. Solutions were generally buffered with 10 mM-Hepes as its K^+ salt, pH 7.0. When 'ghosts' were loaded at pH 6.0, lysis was performed in a solution containing 10 mM-Mes and 0.1 mM-EGTA as their K^+ salts, pH 6.0, and solutions used in purification were also buffered with 10 mM-Mes, pH 6.0. Na^+-loaded 'ghosts' were prepared by lysis of granules in 10 mM-Hepes/0.1 mM-EGTA/KOH (pH 7.0) containing 25 mM-Na_2SO_4; solutions used in subsequent purification then also contained 25 mM-Na_2SO_4. Protein concentrations of 'ghost' suspensions were determined by the method of

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; TMA, tetramethylammonium; 5HT, 5-hydroxytryptamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Bradford (1976), with bovine serum albumin as a standard.

Transport assays. ATP-dependent uptake of 5-hydroxy[^{14}C]tryptamine ([^{14}C]5HT) was determined by filtration of 'ghosts' as described previously (Apps *et al.*, 1980), samples being taken at 20 s intervals over 3 min for initial-rate determinations. Simultaneous accumulation of 5HT and Na^+ was measured by incubating 'ghosts' (0.15 mg/ml) at 30 °C in a medium containing 0.3 M-sucrose, 30 mM-Hepes/KOH, pH 7.0, 6 mM-$ATPK_2$, 3 mM-$MgSO_4$, 20 mM-KI, 100 μM-$^{22}Na_2SO_4$ (3 μCi/ml), 50 μM-[^{3}H]5HT (1 μCi/ml). Samples (100 μl) were removed at intervals to ice-cold 0.3 M-sucrose/30 mM-Hepes/KOH, pH 7.0, containing 10 μM-5HT (2.5 ml). Na_2SO_4 was added to 5 mM before filtration. Filters were washed with 2 × 2.5 ml of ice-cold 0.3 M-sucrose/10 mM-Hepes/NaOH, pH 7.0, and radioactivity was determined by scintillation counting.

For analysis on a sucrose gradient, the incubation mixture (20 min at 30 °C) was centrifuged through a small column (2 cm × 0.9 cm diam.) of Bio-Gel P6DG prepared in a Microfuge tube with a hole in the bottom. The Bio-Gel was equilibrated with cold 0.3 M-sucrose/30 mM-Hepes/KOH, pH 7.0. The 'ghosts' were then applied to a linear gradient (13 ml) of sucrose (0.3–1.3 M) in 30 mM-Hepes/KOH, pH 7.0, and centrifuged at 196000 ***g*** for 150 min at 4 °C in an SW41 rotor in a Beckman ultracentrifuge. Fractions (0.6 ml) were collected, and 0.4 ml portions were added to 2.5 ml of buffered 0.3 M-sucrose for filtration as above. Fraction densities were calculated from refractive-index measurements made on a parallel gradient. Acetylcholinesterase (EC 3.1.1.7) and cytochrome *c* oxidase (EC 1.9.3.1) activities were measured in samples of fractions as described by Phillips (1981).

Accumulation of $^{22}Na^+$ by low-pH 'ghosts' was achieved by diluting 'ghosts' made at pH 6.0 (3–6 mg/ml) 30–40-fold into media at 25 °C at the required pH containing 0.3 M-sucrose, 20 mM-Hepes, 0.1 mM-EGTA/KOH and various concentrations of NaCl in the presence of ^{22}NaCl at 4 μCi/ml. Samples (100 μl) were removed for filtration as above. In some experiments, a drop (8 μl) of 'ghost' suspension was placed on the side of a plastic tube (4 ml, flat-base polypropylene; Sarstedt, Leicester, U.K.) containing 200 μl of incubation medium. Uptake was initiated by mixing the pre-warmed (25 °C) contents on a vortex mixer, and was quenched by rapid addition of 3.5 ml of ice-cold 0.3 M-sucrose/10 mM-Hepes/KOH, pH 7.0, containing 5 mM-Na_2SO_4.

Acidification of Na^+-loaded 'ghosts' was measured by dilution of 'ghosts' into a Na^+-free medium containing 0.3 M-sucrose, 30 mM-Hepes/TMA, pH 7.3, 0.1 mM-EGTA, 50 mM-choline chloride, with 2.5 μM-[^{14}C]-methylamine (0.15 μCi/ml). The final Na^+ concentration in the medium was 1.7 mM. The logarithm of the methylamine concentration ratio (concentration inside/concentration outside) is equal to the magnitude of the pH gradient (outside minus inside).

Na^+/Na^+ exchange was measured by incubating 'ghosts' prepared in 0.3 M-sucrose/10 mM-Hepes/NaOH, pH 7.0 (4 mM-Na^+), at 37 °C in the same medium containing 0.1 mM-EGTA/NaOH for up to 2 h. ^{22}NaCl (1.5 μCi/ml) was added at intervals, and accumulation was measured by filtration. For estimating the accumulated concentrations of Na^+ and amines, we used a value of 3.6 μl/mg for the internal volume of the 'ghosts' (Phillips & Allison, 1978) when using media of low ionic strength, but a value of 3 μl/mg when media were supplemented with salt solutions.

In general, kinetic results presented in this paper are typical experiments from a series performed on several preparations of 'ghosts'. The filtration assays gave results reproducible within ±3% (S.E.M. for 5HT or methylamine), or ±15% for ^{22}Na, in which the ratio of accumulated d.p.m. to background is much lower.

Fluorescence experiments. Assays were performed in media (25 °C; final volume 0.5 ml) containing 0.3 M-sucrose, 10 mM-Hepes and 0.1 mM-EGTA/KOH, pH 7.0. NaCl or LiCl was added, with choline chloride to make up the concentration to 60 mM. ACMA was used as a stock solution (50 μg/ml) in ethanol, and was added to 0.75 μM final concn. 'Ghosts' (internal pH 6.0) were used at 0.10–0.15 mg/ml. Excitation (slit-width 5 nm) was at 420 nm, and emission was measured (slit-width 10 nm) at 525 nm in a Perkin–Elmer 3000 fluorimeter. Amiloride was added from a 10 mM stock in water, and ethylisopropylamiloride from a 5 mM stock in ethanol.

All experiments were performed on several preparations of 'ghosts'. Representative traces are presented in this paper.

Alternatively, Na^+-loaded 'ghosts' were added to media containing 0.3 M-sucrose, 30 mM-Hepes, 12 mM-TMA, 50 mM-choline chloride and 0.75 μM-ACMA, pH 7.30, and quenching of ACMA fluorescence was monitored. Responses were calibrated (Warnock *et al.*, 1982) by diluting 'ghosts' loaded with Mes at pH 6.0 into media containing 0.3 M-sucrose, 30 mM-Hepes, 0.1 mM-EGTA at various pH values, adjusted with TMA hydroxide. Results were plotted according to eqn. (1):

$$\log\left(\frac{A_i}{A_o}\right) = \Delta pH + \log\left(\frac{v_i}{v_o}\right) \quad (1)$$

where A_i and A_o are the total amounts of ACMA inside and outside the 'ghosts', ΔpH is the difference in pH (outside minus inside) across the 'ghost' membrane, and v_i and v_o are the volumes of the intra- and extra-vesicular spaces. A_i is measured by the percentage of fluorescence quenched and A_o by the initial fluorescence (100% value) minus the percentage quenched.

Measurements of percentage quenching were then used for determination of ΔpH. The validity of this approach was checked by adding 2 M-HCl to cuvettes in an amount that eliminated quenching, and then measuring the pH of the medium: fair agreement was found between the two methods.

RESULTS

Na^+ inhibition of 5-HT accumulation

Experiments on catecholamine transport by chromaffin granules generally utilize resealed membrane 'ghosts', rather than intact granules, which tend to lyse or to leak their constituents during incubations (Phillips, 1974*a*). Such 'ghosts' have commonly been prepared in media buffered with Hepes in the form of its Na^+ salt, but we have recently found that Na^+ is mildly inhibitory in these transport assays.

5HT is an excellent substrate for the amine transporter, with a K_m of 5–8 μM (Phillips, 1974*b*; Carty *et al.*, 1985).

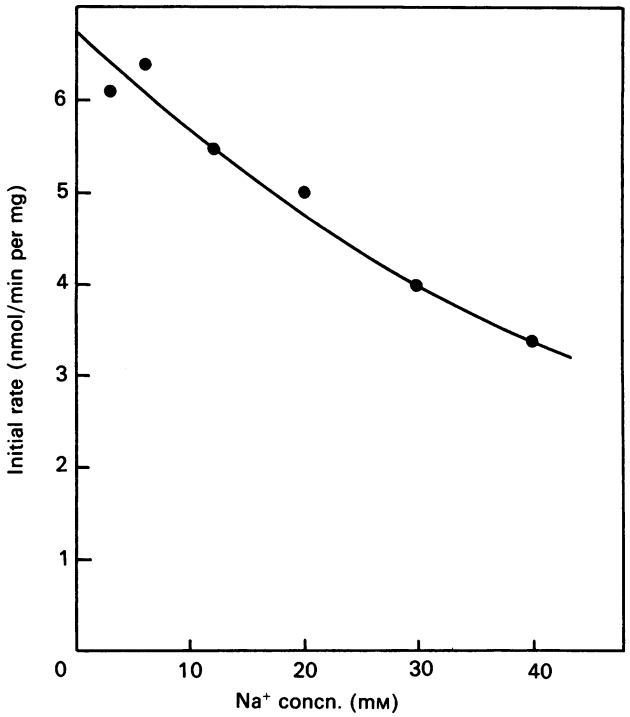

Fig. 1. Na^+ inhibition of 5HT accumulation by 'ghosts'

Chromaffin-granule 'ghosts' (0.11 mg/ml) were incubated with 50 μM-[^{14}C]5HT and MgATP in media supplemented with various concentrations of Na_2SO_4; osmolarity was maintained constant by addition of K_2SO_4. Initial rates of accumulation of 5HT at 30 °C were measured at each Na^+ concentration by filtering samples at intervals of 20 s.

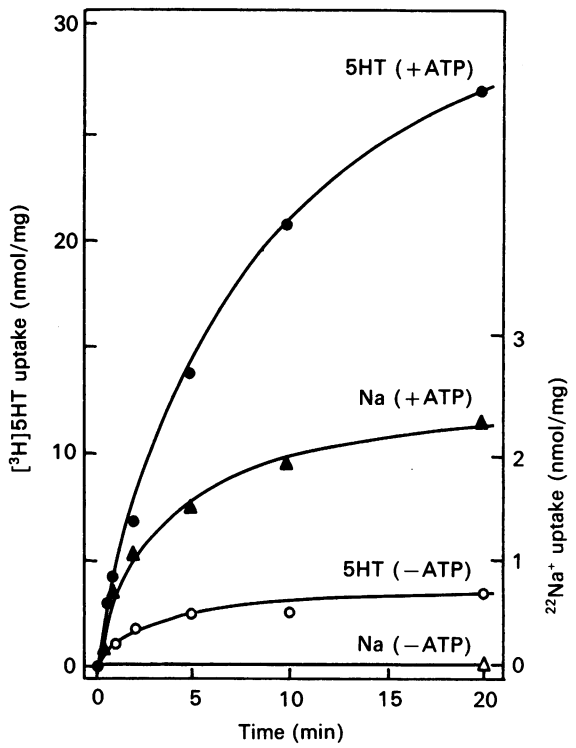

Fig. 2. Accumulation of Na^+ and 5HT by 'ghosts'

Chromaffin-granule 'ghosts' (0.15 mg/ml) were incubated at 30 °C in a standard medium supplemented with 20 mM-KI, 50 μM-[^{3}H]5HT (●, ○) and 100 μM-$^{22}Na_2SO_4$ (▲, △). Incubations were performed in the presence (●, ▲) or absence (○, △) of MgATP.

We have re-investigated the initial rates of its transport into resealed 'ghosts' by using various concentrations of Na^+ in the incubation media, with K^+ present to maintain the ionic strength (Fig. 1). Na^+ proves to be weakly inhibitory. The amine transport is linked to the protonmotive force generated across the 'ghost' membrane by ATP hydrolysis by a process that involves exchange of amine for a proton (Njus *et al.*, 1987*a*). The activity of the proton-translocating ATPase, however, is unaffected by Na^+ concentrations below 50 mM. These observations therefore suggested that Na^+ might have a weak uncoupling role in this system, and we show below that this results from an initial decrease in the proton gradient by exchange of H^+ with Na^+.

ATP-driven Na^+ transport

The establishment of a transmembrane pH gradient in the 'ghosts' can be used to drive accumulation of $^{22}Na^+$. The Na^+/H^+ antiporter has a relatively high K_m for Na^+, so that such experiments are difficult, the accumulation of the radioactive $^{22}Na^+$ being masked by non-radioactive Na^+; the experiments are thus performed at Na^+ concentrations that are well below K_m. In the experiment shown in Fig. 2, 'ghosts' were incubated with MgATP, 20 mM-KI (to increase ΔpH at the expense of $\Delta\psi$), 50 μM-[^{3}H]5HT and 100 μM-$^{22}Na_2SO_4$, so that the use of the proton gradient for amine and Na^+ transport could be examined simultaneously.

Fig. 2 shows that Na^+ equilibrates across the membrane. Osmotic lysis released both 5HT and Na^+ (results not shown), and the uptake of both is dependent on MgATP. After about 20 min the plateau value of accumulated Na^+ (about 2.5 nmol/mg) corresponds to an internal concentration of approx. 0.8 mM, assuming an internal volume of about 3 μl/mg of protein, slightly less than the value measured by Phillips & Allison (1978) at lower osmolarity. This is equivalent to a concentration gradient of about 8-fold across the membrane. In contrast, 5HT uptake is far more extensive and proceeds

Table 1. Effects of inhibitors on Na^+ and 5HT accumulation by 'ghosts'

'Ghosts' were incubated with [^{3}H]5HT and $^{22}Na^+$ as described in Fig. 2. Values given are related to control values (in the presence of ethanol if appropriate) after 20 min incubation, and are means ± S.E.M. (n = 3).

Addition to medium	Accumulation (% of control value) 5HT	Na^+
Ethanol (0.5%)	111±2.6	120±9.0
$(NH_4)_2SO_4$ (10 mM)	1±0.7	31±5.5
FCCP (10 μM)	19±1.0	62±3.2
Ouabain (1 mM)	102±5.9	106±14.2
Sodium orthovanadate (10 μM)	125±4.7	109±1.6
N-Ethylmaleimide (30 μM)	32±2.7	30±4.6
Reserpine (10 μM)	10±3.0	90±9.2
Amiloride (1 mM)	105±4.0	68±12.2

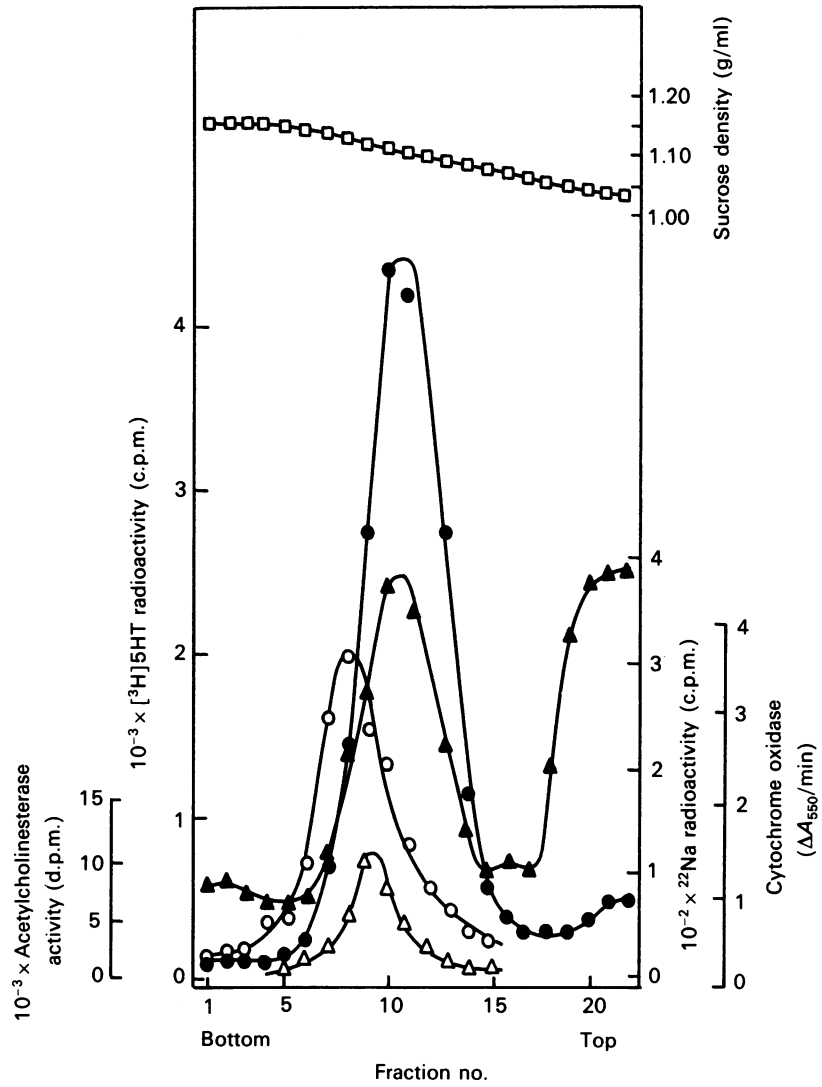

Fig. 3. Sucrose-gradient analysis of 'ghosts'

Chromaffin-granule 'ghosts' were incubated with MgATP, [^{3}H]5HT and $^{22}Na_2SO_4$ as described in Fig. 2. After 20 min at 30 °C they were analysed on a sucrose gradient. Portions of the fractions collected were filtered and analysed for radioactivity ([^{3}H]5HT, ●; ^{22}Na, ▲) or were assayed for acetylcholinesterase (△) or cytochrome oxidase (○) activities. Refractive-index measurements made on a parallel gradient were used for calculation of fraction densities (□).

for longer, a concentration gradient of about 200-fold being demonstrated in Fig. 2. The high concentration gradient achieved with the amine (Phillips & Apps, 1980) results from the electrogenic exchange of two protons for each amine cation that is catalysed by the amine transporter (or, more probably, the exchange of one proton for an uncharged amine molecule; Scherman & Henry, 1981). It achieves equilibrium with the proton electrochemical gradient more slowly than the Na^+/H^+ exchange.

We used the assay shown in Fig. 2 to examine the effects of some inhibitors on the two transport systems (Table 1). It was too difficult to measure initial rates accurately, so that accumulation at 20 min is given in Table 1. Under these conditions, 5HT uptake was 23.2 ± 1.72 nmol/mg (S.E.M. for the three different 'ghost' preparations) and Na^+ uptake was 1.85 ± 0.29 nmol/mg. Transport of both 5HT and Na^+ was decreased by inclusion of the uncoupler FCCP, by NH_4^+ (which decreases the pH gradient across the membrane), and by *N*-ethylmaleimide, an inhibitor of the H^+-ATPase (Percy & Apps, 1986). Neither substrate was affected by ouabain or vanadate, which do not affect this ATPase (Percy *et al.*, 1985). Reserpine is a potent inhibitor of the amine transporter, but also has some detergent-like effect on the 'ghosts' (Zallakian *et al.*, 1982). Amiloride inhibits the accumulation of Na^+.

We also used this assay method (simultaneous incubation of 'ghosts' with [^{3}H]5HT and $^{22}Na^+$ in the presence of ATP) to show that the Na^+ is accumulated into the same compartment as the amine. After the incubation, we subjected the 'ghosts' to equilibrium sedimentation on a sucrose density gradient. The 'ghosts' used in these experiments are already highly purified, by a method (Apps *et al.*, 1980) that is itself based on density-gradient centrifugation, so that contaminating organelle fragments are very close in density to the 'ghosts' themselves. Contaminants can, however, be resolved from 'ghosts' on a further gradient, and the dual-label technique is used as a sensitive assay for co-equilibration.

The results shown in Fig. 3 demonstrate that $^{22}Na^+$ is

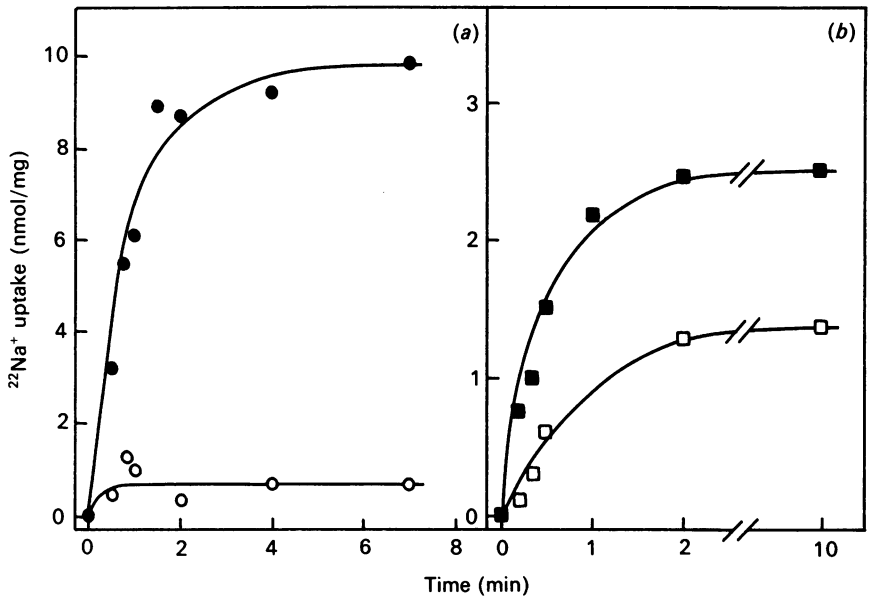

Fig. 4. Accumulation of Na^+ by acid-loaded 'ghosts'

Chromaffin-granule 'ghosts' loaded with buffer at pH 6.0 were diluted to a concentration of 0.2 mg/ml, into media at 30 °C as described under 'Methods'. These media contained (*a*) 1.0 mM-$^{22}NaCl$ (4 μCi/ml) with final pH values of 8.0 (●) and 6.0 (○), or (*b*) 0.5 mM-$^{22}NaCl$ (4 μCi/ml) at pH 7.0, without (■) or with 1 mM-amiloride (□).

co-sedimenting with the incorporated amine. Radioactivity at the top of the gradient (similar amounts of [^{3}H]5HT and $^{22}Na^+$) is not in particulate material, but results from some carry-over of the radioisotopes from the incubation medium (or, possibly, leakage from the 'ghosts'). Acetylcholinesterase activity is found in the matrix of intact chromaffin granules (Gratzl, 1984; Burgun *et al.*, 1985), but, when membrane 'ghosts' are used, this activity can be used as a convenient marker for plasma-membrane fragments; both it and cytochrome oxidase (a marker for mitochondria) sediment at slightly higher densities than the 'ghosts', as found in previous studies (Phillips, 1981).

pH-jump experiments

The experiments described above suggested that Na^+ could enter the 'ghosts' in response to the activity of the 'ghost' membrane H^+-ATPase. We have made a direct demonstration that uptake is in response to the pH gradient generated by this ATPase by using 'ghosts' lysed and resealed in the presence of Mes buffered at pH 6.0, and then purified in the usual way. These acid-loaded 'ghosts' were diluted into media at higher pH that contained $^{22}Na^+$, but no ATP. There is a rapid accumulation of $^{22}Na^+$ (Fig. 4); after about 2 min this reaches a maintained plateau value that depends on the pH gradient. In the experiments shown, the initial pH gradients were 1.0 or 2.0 pH units. The internal Na^+ concentration found at the plateau value when $\Delta pH = 1$ was approx. 0.7 mM, in the presence of 0.5 mM-Na^+ in the medium, and when $\Delta pH = 2$ the plateau value was 2.7 mM with 1.0 mM-Na^+ externally.

We attempted to use this method to investigate the kinetics of Na^+ accumulation, using a pH jump from pH 6 to 7, and Na^+ concentrations in the range 0.5–50 mM. The shortest incubation time that it was practicable to use, however, was 10 s, and, as shown in Fig. 4(*b*), the accumulation is non-linear at this point. Estimates of the K_m for Na^+ were of the order of 20–30 mM, however, with $V_{max.}$ values as high as 150–200 nmol/min per mg. We were unable to make more accurate measurements than this.

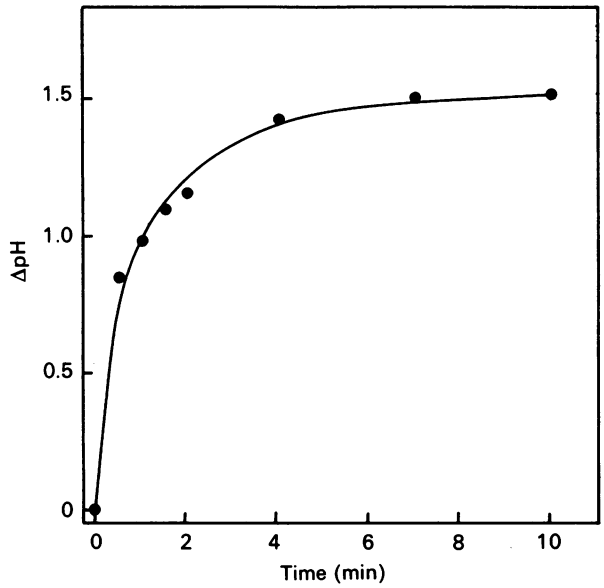

Fig. 5. Generation of a pH gradient by Na^+-loaded 'ghosts'

Chromaffin-granule 'ghosts' containing 25 mM-Na_2SO_4 were diluted into medium at 25 °C to give a protein concentration of 0.12 mg/ml and a final Na^+ concentration of 1.7 mM. 'Ghost' acidification was followed by accumulation of [^{14}C]methylamine and is plotted as the pH gradient (ΔpH) across the membrane.

Fig. 4 also demonstrates that this H^+-driven Na^+ accumulation is sensitive to amiloride, an inhibitor of plasma-membrane Na^+/H^+ exchangers. This is explored further below.

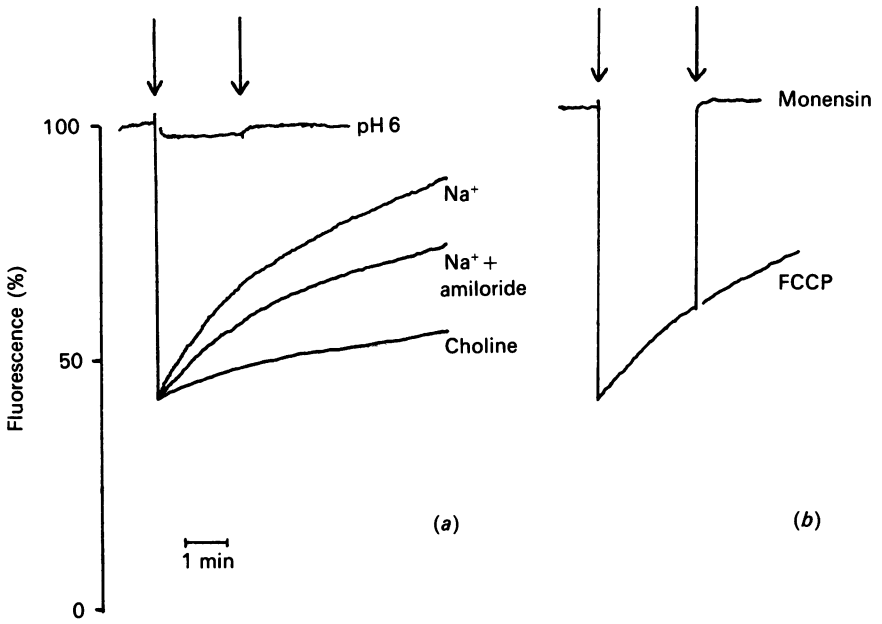

Fig. 6. Demonstration of Na^+ entry into acid-loaded 'ghosts' by using ACMA fluorescence

(*a*) 'Ghosts' loaded at pH 6.0 were diluted into medium at pH 7.0 (25 °C) containing ACMA at the times shown by the first arrow, and fluorescence was recorded. Media were supplemented with 50 mM-choline chloride, 50 mM-NaCl, or 50 mM-NaCl plus 1 mM-amiloride. In a control experiment, the medium was at pH 6.0; 50 mM-NaCl was added at the second arrow. (*b*) As (*a*), with medium containing 50 mM-NaCl; at the second arrow was added either 10 μM-monensin or 10 μM-FCCP. In all cases the final protein concentration was 0.12 mg/ml, and the time and fluorescence scales were as shown in (*a*).

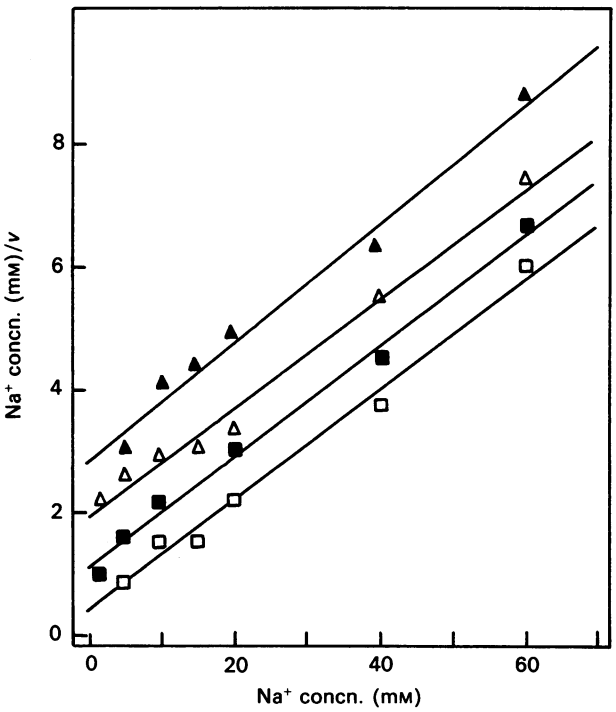

Fig. 7. Competitive inhibition of Na^+/H^+ antiporter by amiloride

Na^+ entry into acid-loaded 'ghosts' was assayed by ACMA fluorescence as described in Fig. 6. Media were supplemented with NaCl and choline chloride to make a total salt concentration of 60 mM. Initial rates of recovery of ACMA fluorescence (v, arbitrary units) were measured in the absence of amiloride (□) or in the presence of (■) 0.5 mM-, (△) 1.0 mM- or (▲) 1.5 mM-amiloride.

Na^+-jump experiments

Reversibility of the exchanger was examined by diluting Na^+-loaded 'ghosts' into medium containing no Na^+ but at the same pH (7.0) as their internal pH. A trace amount of [^{14}C]methylamine was added in order to follow the generation of a pH gradient (ΔpH) across the membrane (acid inside) in response to Na^+ efflux. Such a gradient is in fact established rapidly, and is maintained for a considerable time (Fig. 5). In the experiment shown, a 30-fold gradient of Na^+ across the membrane sustained a pH gradient of 1.5 units (a 30-fold H^+ concentration gradient), suggesting a stoichiometry of the exchanger of 1:1 if it is the only mechanism for linking the two gradients.

Fluorescence assay of Na^+/H^+ exchange

Because of the difficulty noted above of making a kinetic analysis of the exchanger by using ^{22}Na, in view of its high K_m for Na, we used another method for direct monitoring of transmembrane proton movements in response to influx or efflux of Na^+. This employed the fluorescent weak base ACMA.

We first calibrated the quenching of ACMA fluorescence by adding 'ghosts' containing 10 mM-Mes, pH 6.0, to media buffered at various pH values, by using the method described by Warnock *et al.* (1982). ACMA accumulation by the 'ghosts' in response to the pH gradient leads to quenching of fluorescence, and this is a function of the magnitude of the pH gradient. We plotted this according to eqn. (1), obtaining a straight-line plot with a slope of 0.6. The deviation from the ideal value of 1.0 may be ascribed to dye binding to the membranes (Warnock *et al.*, 1982). The measured slope was found to

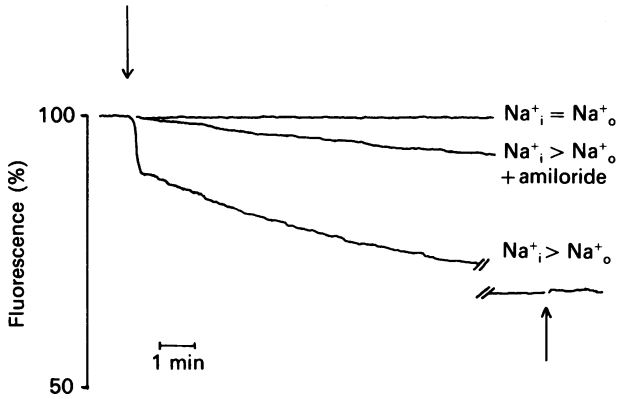

Fig. 8. Acidification of Na^+-loaded 'ghosts'

'Ghosts' loaded with 25 mM-Na_2SO_4 at pH 7.0 were diluted into medium (pH 7.0, 25 °C) containing ACMA at the time shown by the first arrow to give a concentration of 0.12 mg/ml, and fluorescence was monitored. In the control experiment, the medium contained 25 mM-Na_2SO_4 so that no Na^+ gradient was established. In the other experiments the final medium contained 0.85 mM-Na_2SO_4 (Na^+_o = 1.7 mM), with or without 1 mM-amiloride. At the second arrow (30 min after the start), either 10 μM-monensin or 10 μM-FCCP was added.

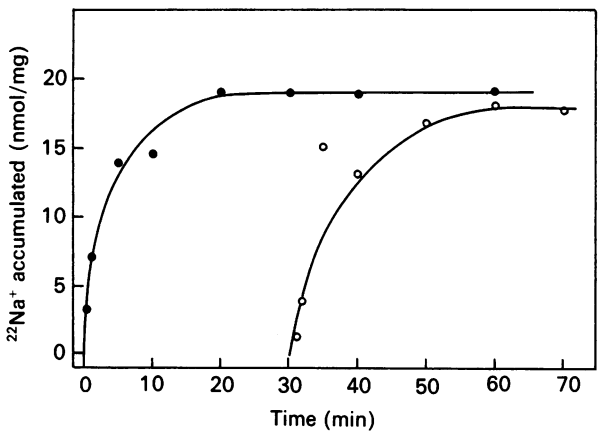

Fig. 9. Na^+/Na^+ exchange by 'ghosts'

'Ghosts' containing 4 mM-Na^+ were incubated at 37 °C in a medium with a similar concentration of Na^+ (5 mM) at a protein concentration of 0.12 mg/ml. The medium was supplemented with $^{22}NaCl$ (1.5 μCi/ml) either at the start of the incubation (●) or after 30 min (○), and $^{22}Na^+$ accumulation was monitored by filtration of 100 μl samples.

vary depending on the cation present in the medium, so conditions for calibration curves mimicked experiments as closely as possible.

The quenched fluorescence is restored if Na^+ is present in the medium (Fig. 6), as Na^+ ions enter the 'ghost' in exchange for H^+. As shown in Fig. 6(*a*), there is some leakage of protons if Na^+ is replaced by choline. This technique can be used, however, to measure the dependence of proton efflux rate on concentration of Na^+, and thus to derive an apparent K_m for Na^+ (Fig. 7): we have found a value of 4.7 ± 0.6 mM (mean ± S.E.M. for five independent determinations), when using 'ghosts' made with internal pH 6.0, in a medium at pH 7.0. Fig. 6 shows the lack of both quenching and Na^+ effects in the absence of a pH gradient, and (Fig. 6*b*) the effect of the ionophore monensin (catalysing the rapid exchange of Na^+ for H^+), and the lack of effect of the electrogenic protonophore FCCP.

As also shown in Fig. 6(*a*), and analysed in Fig. 7, this technique can be used for the investigation of inhibitors of the exchanger. Amiloride is found to be a competitive inhibitor with K_i 0.26 ± 0.02 mM (mean ± S.E.M. for three amiloride concentrations). We also investigated the amiloride derivative ethylisopropylamiloride; however, this was found to have an uncoupling effect in this system, collapsing ΔpH in a concentration-dependent manner in the absence of Na^+. This was manifested as a decrease in ACMA quenching when it was present at 10–100 μM, and an increased rate of proton efflux in the presence of choline chloride (under conditions of Fig. 6). At low concentrations (up to 50 μM), however, it appeared to have no inhibitory effect on Na^+ entry. In assays of 5HT transport, 30 μM-ethylisopropylamiloride was found to decrease the initial rate of uptake by 70%, consistent with its facilitating proton leakage.

Although not shown, the ACMA-quenching technique can also be used to investigate alternative substrates, by replacing Na^+ with other cations. K^+ cannot replace Na^+, but Li^+ is a weak alternative substrate. Initial rates with Li^+ are low, however. The K_m for Li^+ was approx. 6 mM with a maximum initial rate about one-fifth that found with Na^+.

A similar approach can be used to measure H^+ entry in response to Na^+ efflux (Fig. 8). 'Ghosts' containing 50 mM-Na^+ were diluted into Na^+-free medium containing ACMA, and the resulting fluorescence quenching was monitored. The experiment shown in Fig. 8 was performed under conditions very similar to those of the experiment recorded in Fig. 5. In Fig. 8 the 30-fold gradient of Na^+ sustains a pH gradient of approx. 1.5 units, as measured by the percentage quenching of the ACMA.

Equilibrium is reached after about 10–20 min, and is then sustained for at least a further 30 min under these conditions. At equilibrium, addition of either monensin or FCCP has no effect on the trace, although the quenching is eliminated by adding either H^+ or Na^+ to the external medium.

Proton entry was found to be sensitive to amiloride (Fig. 8). Obviously one cannot tell from this experiment whether the drug is penetrating into the lumen of the 'ghosts' or is acting on their outer surface.

Na^+/Na^+ exchange

'Ghosts' that contain Na^+ exchange this for $^{22}Na^+$ in the medium: this is probably a function of the Na^+/H^+ antiporter, already shown to be reversible. This is demonstrated in Fig. 9. 'Ghosts' containing approx. 4 mM-Na^+ were incubated in medium containing 5 mM-$^{22}Na^+$, which is accumulated over a period of about 30 min. A similar incubation was performed in the presence of the same concentration of non-radioactive Na^+. At 30 min, when, according to the first result, $^{22}Na^+$ uptake had essentially ceased, a trace amount of $^{22}Na^+$ was added to the second incubation. Uptake of radioactivity occurred as in the first incubation, showing that at 30 min there is an equilibrium, with the rate of loss of Na^+ from the 'ghosts' equalling the rate of uptake, the $^{22}Na^+$ having equilibrated across the membrane. The plateau value of $^{22}Na^+$ reached in Fig. 9

(18–19 nmol/mg) is equivalent to an internal concentration of 5 mM, if the internal volume of the 'ghosts' is 3.6 μl/mg (Phillips & Allison, 1978).

Amiloride inhibited Na^+/Na^+ exchange (50 % decrease in initial rate by 0.5 mM-amiloride; results not shown). This strongly suggests that the exchange is indeed catalysed by the Na^+/H^+ antiporter.

DISCUSSION

We have assayed a novel Na^+/H^+ exchange activity of chromaffin-granule membranes by employing a variety of techniques: direct assay of $^{22}Na^+$ accumulation in response to a pH gradient generated by pH jump or by ATP hydrolysis; generation of a pH gradient in response to an imposed Na^+ gradient; loss of a pH gradient resulting from Na^+ uptake; and Na^+/Na^+ exchange. The antiporter has a relatively high K_m for cytosolic (extragranular) Na^+. A K_m value of 4.7 mM at pH 7.0 was determined from fluorescence experiments (an indirect measurement of Na^+ transport), with values closer to 20 mM being derived from assays using $^{22}Na^+$. $V_{max.}$ values determined from $^{22}Na^+$ transport rates in pH-jump experiments in the absence of ATP were of the order of 150–200 nmol/min per mg; such rates are sustained for only a few seconds, presumably because of rapid loss of the trans-membrane pH gradient. For comparison, $V_{max.}$ of the H^+-ATPase of the granule is about 100–200 nmol/min per mg of membrane protein (Johnson *et al.*, 1982; Percy *et al.*, 1985), and that of the catecholamine translocator is about 15 nmol/min per mg (Carty *et al.*, 1985).

Interaction between the Na^+ and H^+ gradients of the 'ghosts' is demonstrated by the inhibitory effect of Na^+ on the initial rate of 5HT transport (Fig. 1). Presumably, when the proton gradient is established from zero as a result of ATP hydrolysis, the presence of Na^+ outside the 'ghosts' facilitates proton leakage and hence a decrease in the driving force of 5HT uptake.

The stoichiometry of the exchanger appears to be 1:1. Exchange is unaffected by ionophores that generate potential gradients (e.g. FCCP), so is probably non-electrogenic, and the approximate measurements of equilibrium H^+ and Na^+ gradients in Figs. 5 and 8 suggest fulfilment of the condition

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[Na^+]_i}{[Na^+]_o} \quad (2)$$

for the two ion gradients. In these experiments an initial Na^+ gradient of about 30-fold supported formation of H^+ gradients of about the same magnitude, as measured by both methylamine distribution and ACMA quenching. The size of the Na^+ gradient at equilibrium has not been determined in these experiments, but rough measurements suggest that it is not much displaced from the initial value. In contrast with H^+ entry, Na^+ uptake by the 'ghosts' in response to a pH jump (Fig. 4) produced plateau values of Na^+ that were well below those predicted by eqn. (2). This may result from rapid loss of the pH gradient, but even the control values in Fig. 4(*a*) are less than would be expected. This clearly merits a more detailed investigation.

It is not, of course, known whether this equilibrium condition (Eqn. 2) is met *in vivo*, but it seems likely that it is, in view of the relatively high capacity of the exchanger. If the pH gradient across chromaffin-granule membranes in intact cells is about 1.5 pH units (acid inside), a 30-fold gradient of Na^+ activity from granule matrix to cytosol could be sustained. The cytosolic concentration of Na^+ is unknown, but is presumably of the order of 1–5 mM; the activity coefficient of Na^+ in the matrix is also unknown, though is likely to be less than unity (Koppell & Westhead, 1982). The intra-granular concentration of Na^+ has been measured by Krieger-Brauer & Gratzl (1982) to be about 47 mM, a value that, though subject to considerable error, would be consistent with the idea that the gradients are in equilibrium.

Ornberg *et al.* (1988) have reported an electron-probe microanalysis of cultured bovine adrenal cells; they suggest that the high granule content of Na^+ reported by Krieger-Brauer & Gratzl (1982) is an artefact arising from exchange of intragranular K^+ for Na^+ *post mortem* and during granule isolation, and that the granule matrix is free of Na^+ *in situ*. This seems unlikely from our results, but clearly needs to be investigated further, in view of the low concentrations of Na^+ found in 'ghosts' at equilibrium in Fig. 4.

It therefore seems that these secretory vesicles have two transmembrane ion gradients that can be used as energy sources: the H^+ gradient that is utilized directly in catecholamine transport, and a Na^+ gradient that is coupled to Ca^{2+} transport (Phillips, 1981; Krieger-Brauer & Gratzl, 1982). The granule's membrane potential facilitates electron transport (Njus *et al.*, 1987*b*) and, probably, nucleotide transport (Weber & Winkler, 1981).

Is the Na^+/H^+ antiporter identical with the plasma-membrane Na^+/H^+ exchanger (reviewed by Aronson, 1985) that has been identified in many cell types? This activity plays a key role in regulation of cytosolic pH, and, through this, of other physiological functions. The specificity and K_m values of the carriers appear similar, as is the K_i value for amiloride (Lazdunski *et al.*, 1985; Seiler *et al.*, 1985). The main difference appears to be the lack of inhibition of the granule exchanger by ethylisopropylamiloride, a highly potent inhibitor of the plasma-membrane activity, showing, for example, a K_i of 22 μM for exchange by dog cardiac sarcolemmal vesicles (Seiler *et al.*, 1985). This clearly needs further investigation.

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