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Transport of Ca^{2+} and Na^+ across the chromaffin-granule membrane

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Bovine chromaffin-granule ghosts accumulate $^{45}Ca^{2+}$ in a temperature- and osmotic-shock-sensitive process; the uptake is saturable, with K_m 38 μM and $V_{max.}$ 28 nmol/min per mg at 37°C. Entry occurs by exchange with Ca^{2+} bound to the inner surface of the membrane. It is inhibited non-competitively by Na^+, La^{3+} and Ruthenium Red (K_i 10.7 mM, 7 μM and 2 μM respectively), and competitively by Mg^{2+} (K_i 0.9 mM). Uptake was not stimulated by ATP. Na^+ induces Ca^{2+} efflux; Ca^{2+} can re-enter the ghosts by a process of Ca^{2+}/Na^+ exchange. La^{3+} inhibits Ca^{2+} efflux during Ca^{2+}-exchange, and Ca^{2+} efflux induced by Na^+, suggesting that Ca^{2+} uptake and efflux, and Ca^{2+}/Na^+ exchange, are catalysed by the same protein. Na^+ enters ghosts during Ca^{2+} efflux, but the kinetics of its entry are not exactly similar to the kinetics of Ca^{2+} efflux. Initially 1–2 Na^+ enter per Ca^{2+} lost, but at equilibrium 3–4 Na^+ have replaced each Ca^{2+}. There is no evidence that either Ca^{2+} uptake or efflux by Ca^{2+}/Na^+ exchange is electrogenic, suggesting that the stoichiometry of exchange is $Ca^{2+}/2Na^+$. This exchange reaction may have a role in depleting cytoplasmic Ca^{2+} after depolarization-induced Ca^{2+} entry through the adrenal medulla plasma membrane; there is some evidence that there may be an additional entry mechanism for Na^+ across the granule membrane.

It is well known that chromaffin granules, the storage granules of the adrenal medulla, contain catecholamines and ATP at high concentrations. Borowitz *et al.* (1965) showed that Ca^{2+} is also a major component; the matrix of the granule contains 20–30 mM-Ca^{2+}, and this accounts for about 60% of that found in the whole tissue (Phillips *et al.*, 1977). The high intra-granular concentration is comparable with that estimated within certain mitochondria (Baker *et al.*, 1971; Nicholls & Scott, 1980) or within sarcoplasmic reticulum (MacLennan & Wong, 1971).

The function of chromaffin granule Ca^{2+} is uncertain. It has been suggested that its role is the stabilization of catecholamine–ATP complexes (Pletscher *et al.*, 1974), but n.m.r. studies have provided no evidence that these complexes do in fact exist within the granules (Sharp & Richards, 1977; Sen *et al.*, 1979). Another possibility is that the granules are a reservoir for Ca^{2+} that penetrates the plasma membrane during the secretory process (Serck-Hanssen & Christiansen, 1973); this is an attractive theory in view of the large number of granules within each cell and the high proportion of cell Ca^{2+} that is stored within them. It is also of interest in view of the high concentrations of Ca^{2+} found within other secretory granules, such as the zymogen granules of the exocrine pancreas (Clemente & Meldolesi, 1975).

Kostron *et al.* (1977) demonstrated that intact chromaffin granules accumulate $^{45}Ca^{2+}$ *in vitro*. Their data suggested that this was a carrier-mediated process, but, because of the high endogenous calcium content of the granules, these workers were unable to investigate the transport mechanism. Uptake was not stimulated by ATP; however, the free Ca^{2+} activity in the matrix is presumably very much below its total concentration. Most is bound to ATP (internal concentration 130 mM), to acidic internal proteins (total matrix protein concentration 180 mg/ml) and to the inner surface of the granule membrane. The work of Johnson & Scarpa (1976) on Ca^{2+} movements into and out of granules in the presence of ionophores suggests that the free Ca^{2+} concentration within the granules is in the low micromolar range. Transport studies with intact granules, however, are hampered by leakage of the endogenous Ca^{2+} during incubations and by the possibility of exchange processes being catalysed by the carrier. To some extent it is possible to

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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circumvent this problem by using resealed chromaffin-granule membranes ('ghosts'), as shown in the present study.

Experimental

Materials

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hepes and biochemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., and other chemicals were from BDH Chemicals, Poole, Dorset, U.K. Ionophore A23187 was a gift from Eli Lilly, Indianapolis, IN, U.S.A., and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was from the Boehringer Corporation (London), Lewes, East Sussex, U.K. Ethanolic solutions of the ionophores were used, but the concentration of ethanol in incubations did not exceed 1%.

Sephadex G-50 (medium) was from Pharmacia (Great Britain), Hounslow, Middlesex, U.K., and cellulose nitrate filters were obtained from Sartorius, Göttingen, Germany. Ruthenium Red, from Sigma, was purified as described by Luft (1971) before use and was estimated by its absorbance at 533 nm. All solutions used were buffered with Tris/Hepes (unless otherwise stated); the pH of 1 M-Hepes (free acid) was adjusted by addition of 2 M-Tris base. EGTA solutions were brought to pH 7.0 with Tris base.

Methods

Resealed chromaffin granule ghosts were prepared by lysis of crude bovine chromaffin granules as described previously (Apps *et al.*, 1980), except that all solutions were buffered with 10 mM-Tris/Hepes, pH 7.0, instead of sodium Hepes. The 'crude ghosts' used in one experiment in Fig. 2 were lysed chromaffin granules that had not been subjected to density gradient centrifugation to purify them from mitochondrial contamination.

Accumulation of Ca^{2+} and Na^+ was measured as follows: ghosts (100–200 μg/ml) were incubated in 0.3 M-sucrose containing 10 mM-Tris/Hepes, pH 7.0, and $^{45}CaCl_2$ or $^{22}NaCl$ (1.0–2.5 μCi/ml) and non-radioactive $CaCl_2$ or Na_2SO_4 as indicated in the Figures. Samples (100 μl) were removed at intervals to 2.5 ml of ice-cold 0.3 M-sucrose containing 10 mM-Tris/Hepes, pH 7.0. For $^{45}Ca^{2+}$ accumulation, this solution also contained 1 mM-EGTA. After having been left on ice for a few minutes, the solutions were filtered directly, or, for $^{22}Na^+$ accumulation, after addition of Na_2SO_4 to 4 mM, through 0.45 μm membrane filters. These were washed with 2 × 2.5 ml of cold 0.3 M-sucrose containing 10 mM-Tris/Hepes or 10 mM-sodium Hepes respectively, and then dried and counted for radioactivity in scintillation fluid containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene.

The sucrose-gradient experiment described in Fig. 2 was performed as follows. Ghosts (200 μg in 0.7 ml) were incubated for 10 min at 37°C with 140 μM-$^{45}CaCl_2$ (sp. radioactivity 19 Ci/mol). To this solution were then added 59 μM-5-hydroxy-[^{3}H]tryptamine (sp. radioactivity 50 Ci/mol), 2.9 mM-$MgSO_4$ and 5.9 mM-ATP (sodium salt). After a further 5 min at 37°C the suspension was cooled on ice and passed down a column (0.7 cm × 8.0 cm) of Sephadex G-50 equilibrated with 0.3 M-sucrose containing 10 mM-Tris/Hepes, pH 7.0. Ghosts emerged in the void volume and were applied (0.35 ml) to a gradient (4.5 ml) of sucrose (0.3–1.5 M) in the same buffer. This was centrifuged for 2 h at 4°C at 200000 ***g*** (Beckman rotor SW50.1). Fractions were collected, and 200 μl samples of each were transferred to 2.5 ml of cold 0.3 M-sucrose containing 10 mM-Tris/Hepes and 1 mM-EGTA; solutions were then filtered as above. Densities of fractions were estimated from measurements of refractive index. Parallel gradients were run with ghosts that had been incubated (40 min at 37°C) in a medium containing 0.75 mM-EGTA, 0.5 mM-Na_2SO_4 and $^{22}NaCl$ (3.8 μCi/ml), and with crude ghosts, as described above. Samples (20 μl) of fractions were assayed for acetylcholinesterase (EC 3.1.1.7) by a modification of the method of Potter (1967) using 45 μM-[*acetyl*-^{3}H]acetylcholine (sp. radioactivity 80 Ci/mol) as substrate; 40 μl samples were assayed for cytochrome *c* oxidase (EC 1.9.3.1) by the method of Mason *et al.* (1973).

Calcium was determined by atomic-absorption spectrophotometry as described previously (Phillips *et al.*, 1977). Protein was determined by the method of Bradford (1976). When results are quoted as means, standard deviations are given and the number of independent experiments is indicated in parentheses.

Results

Calcium accumulation by ghosts

Lysis of intact chromaffin granules by osmotic shock releases all but about 15% of their calcium (Phillips *et al.*, 1977). Residual calcium remains bound to the membranes, however; calcium determinations on four preparations of chromaffin-granule ghosts gave a mean content of 67 ± 14 nmol/mg of protein. Removal of calcium from sucrose solutions before purification of the ghosts did not greatly decrease this value, and was therefore omitted.

Incubation of the ghosts in sucrose medium containing $^{45}Ca^{2+}$ leads to a rapid incorporation of calcium (Fig. 1). The initial high rate observed at 37°C is maintained for less than 30s; a steady

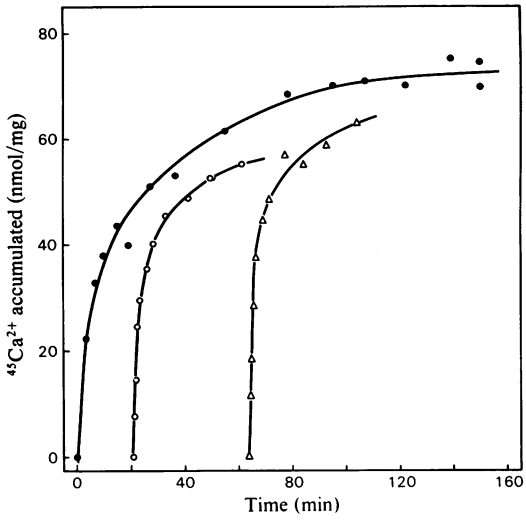

Fig. 1. *Accumulation of Ca^{2+} by chromaffin-granule ghosts*

Ghosts were incubated at 37°C in buffered sucrose containing 150 μM-$^{45}CaCl_2$ (2.4 μCi/ml), and samples were withdrawn at intervals for assessment of $^{45}Ca^{2+}$ accumulation (●). Two parallel incubations were maintained under identical conditions except that the $CaCl_2$ was non-radioactive; $^{45}CaCl_2$ (2.4 μCi/ml) was added to one at 21 min (○) and the other at 64 min (△) after the start of the incubation.

uptake is observed, however, with gradual approach to a plateau value. The incorporation is assayed by withdrawing samples and adding them to an excess of cold buffered sucrose containing EGTA. This serves to chelate the radioactive Ca^{2+} in the medium and to remove Ca^{2+} bound to the external surface of the ghosts (Reed & Bygrave, 1975). Accumulated Ca^{2+} is retained owing to the low permeability of the ghost membrane at 0°C, and can be assayed after removal of the ghosts by filtration. If sucrose is omitted from the quenching solution (which contains EGTA), 70% of the $^{45}Ca^{2+}$ is released, owing to the osmotic shock received by the ghosts. Presumably the remaining tracer is firmly bound inside the ghosts and does not dissociate before membrane resealing occurs.

The plateau value reached (72 nmol of $^{45}Ca^{2+}$/mg of protein taken up from the medium in the example shown in Fig. 1) is maintained for several hours. It is in fact an equilibrium situation, with the rate of Ca^{2+} influx equalling its rate of efflux. This is shown (Fig. 1) by incubating the ghosts under identical conditions but in the presence of non-radioactive Ca^{2+}. When tracer amounts of $^{45}Ca^{2+}$ are added at 21 and 64 min, uptake of tracer is observed at a rate identical with that seen on initiation of uptake by the ghosts in the original radioactive medium. Since uptake rates are high, but the total radioactive calcium reaches a plateau value, there must be an equally high efflux rate.

Under these conditions we are therefore observing a Ca^{2+} exchange process across the membrane. The osmotic sensitivity of most of the trapped Ca^{2+}, and its insensitivity to external EGTA, suggest that it is located in the interior of the ghosts. The accumulation is markedly temperature-sensitive; the initial rate is decreased to about 30% at 20°C, and is less than 4% at 0°C.

Membrane location of transported Ca^{2+}

It is well known that Ca^{2+}-transport mechanisms are located in the plasma membrane, endoplasmic reticulum and mitochondrial inner membrane. The chromaffin-granule-ghost preparation used in this work is virtually free of marker enzyme activities for other subcellular fractions (Phillips, 1974), although it does contain some plasma-membrane fragments as revealed by the presence of acetylcholinesterase activity.

Ghosts that had accumulated $^{45}Ca^{2+}$ were incubated with [^{3}H]5-hydroxytryptamine and $MgATP^{2-}$; active 5-hydroxytryptamine accumulation was used as a diagnostic marker for the ghosts. They were then centrifuged to equilibrium in a sucrose density gradient (Fig. 2*a*). Fractions from the gradient were assayed by filtration; the main bands of $^{45}Ca^{2+}$ and [^{3}H]5-hydroxytryptamine were coincident. A similar result was obtained when 1 mM-EGTA was included in the gradient, or if the ghosts were centrifuged to equilibrium in an isoosmotic gradient composed of 0.3 M-sucrose and metrizamide (Morris & Schovanka, 1977). About 4% of particle-associated $^{45}Ca^{2+}$ was found in a band equilibrating at higher density. Mitochondrial inner-membrane markers are found in this region if unpurified crude ghosts are centrifuged (Fig. 2*b*). This crude material also contains heterogeneous acetylcholinesterase activity. After ghost purification this activity, presumably associated with plasma-membrane fragments, comes to equilibrium at a slightly higher density than the ghosts (Fig. 2*a*).

These experiments suggest that chromaffin-granule membranes do indeed transport Ca^{2+}, and that the observed incorporation is due to the ghosts and not to contaminating membrane fragments.

Kinetics and inhibition of Ca^{2+} exchange

Ca^{2+} incorporation was found to be a saturable process with K_m $38 \pm 4(5)$ μM and $V_{max.}$ $28 \pm 7(5)$ nmol/min per mg. La^{3+} and Ruthenium Red, inhibitors of Ca^{2+} transport by mitochondria, were found to be non-competive inhibitors, with K_i values 7 μM and 2 μM respectively. Mg^{2+} was a competitive inhibitor with K_i 0.9 mM; at this concentration Mg^{2+} induces sigmoidicity in the kinetics

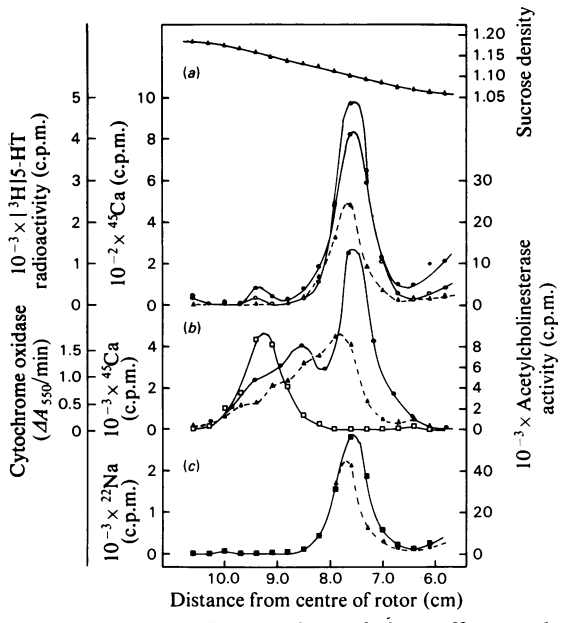

Fig. 2. *Sucrose-gradient analysis of chromaffin-granule ghosts*

(*a*) Ghosts were incubated with $^{45}CaCl_2$, followed by 5-hydroxy[^{3}H]tryptamine ([^{3}H]5-HT) and $MgATP^{2-}$, and then subjected to sucrose-gradient centrifugation as described in the Experimental section. Samples of gradient fractions (200μl) were filtered and assayed for ^{45}Ca (●) and ^{3}H (○), and were also assayed (20μl) for acetylcholinesterase activity (▲). (*b*) Crude ghosts incubated with $^{45}CaCl_2$ were centrifuged, and fractions were filtered and assayed for ^{45}Ca (●); gradient fractions from a parallel gradient were assayed for cytochrome oxidase (40μl; □) and acetylcholinesterase (20μl; ▲). (*c*) Ghosts were incubated with $^{22}Na_2SO_4$ and centrifuged; samples of fractions (200μl) were filtered and assayed for ^{22}Na (■), and were also assayed (20μl) for acetylcholinesterase (▲). Fraction densities (△) were estimated from refractive index measurements.

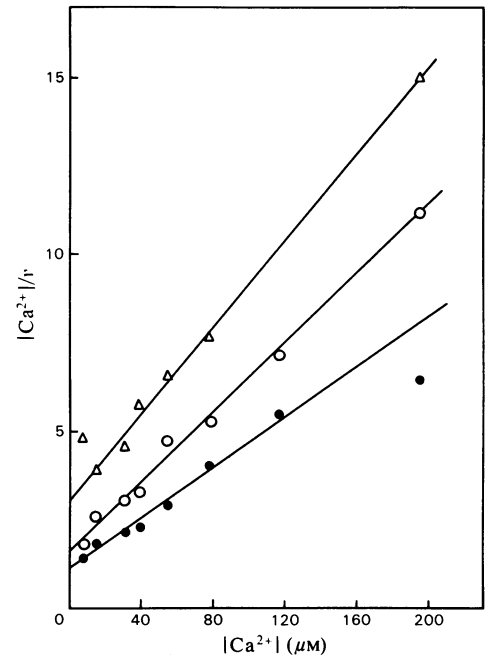

Fig. 3. *Na^+-inhibition of Ca^{2+} uptake by chromaffin-granule ghosts*

Ghosts (160μg/ml) were incubated with $^{45}CaCl_2$ (sp. radioactivity 16.6Ci/mol) at the concentration shown in the absence (●) or presence of 4.55mM-Na^+ (○) or 9.09mM-Na^+ (△) as Na_2SO_4. Samples were assayed for $^{45}Ca^{2+}$ incorporation at 10s intervals. Ca^{2+} concentrations were in μM and initial velocities (v) in nmol/min per mg.

of Ca^{2+} transport by heart mitochondria (Crompton *et al.*, 1976), but no such effect was found with chromaffin granule ghosts. Monovalent ions were also found to inhibit Ca^{2+} exchange (Fig. 3). Na^+ was a non-competitive inhibitor with K_i 10.7mM.

Ca^{2+} transport across many membranes is coupled to ATP hydrolysis, but neither ATP alone nor $MgATP^{2-}$ stimulated uptake by the ghosts. Indeed, they were somewhat inhibitory, presumably because of Ca^{2+} chelation. Various anions were also tested, but uptake was identical in solutions of $CaSO_4$, calcium acetate and $CaCl_2$ (ghost membranes are relatively permeable to chloride, but not to sulphate or acetate; Phillips, 1977).

Ca^{2+}-binding sites

The distribution of $^{45}Ca^{2+}$ in these experiments between free Ca^{2+} within the ghosts and bivalent-cation-binding sites on the inner surface of the ghost membrane is not known. An attempt was made to investigate these Ca^{2+}-binding sites by incorporating ionophores in the membranes, to permit equilibration of free Ca^{2+} between the medium and the internal pool.

Ghosts were incubated as in Fig. 1 in a medium supplemented with 10μM-ionophore A23187 and 5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, the former being a carboxylic bivalent cation ionophore and the latter a mitochondrial uncoupling agent (proton ionophore), to make the membranes freely permeable to Ca^{2+}. Under these conditions Ca^{2+} uptake was rapid, and equilibrium was reached in 20min. The Ca^{2+} concentration in the medium was varied, and that bound within the ghosts at equilibrium was measured by quenching samples at 0°C in EGTA-containing medium, as in Fig. 1. The results are shown as a Scatchard plot in Fig. 4, making the assumption that the Ca^{2+} activity

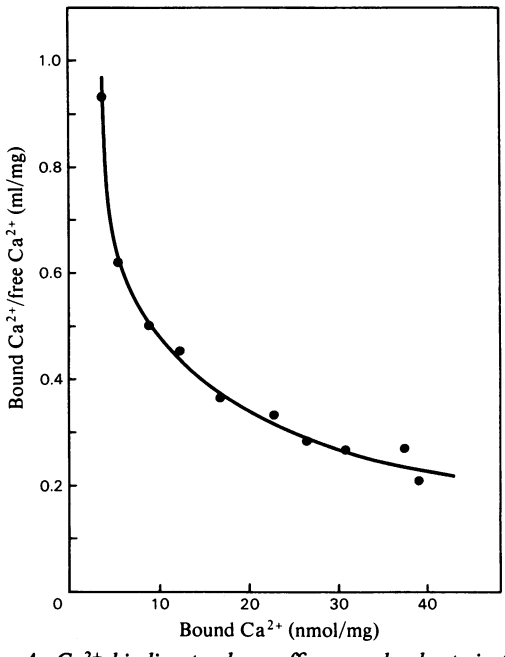

Fig. 4. *Ca^{2+} binding to chromaffin-granule ghosts in the presence of ionophores*

Ghosts (180μg/ml) were incubated for 30min at 37°C in a medium containing $^{45}CaCl_2$ (5–200μM, 24Ci/mol), 10μM-ionophore A23187 and 5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Duplicate samples were filtered as described in the Experimental section, and blank values (filters washed with identical media lacking ghosts) were subtracted. Bound Ca^{2+} (nmol/mg) and free Ca^{2+} (nmol/ml) were calculated from these data.

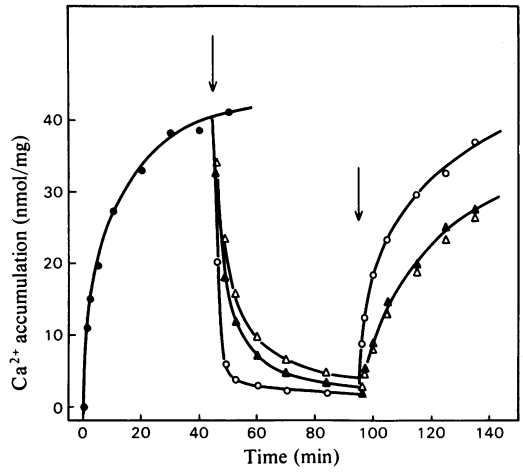

Fig. 5. *Accumulation and EGTA-induced efflux of Ca^{2+}*

Ghosts (140μg/ml) were incubated with 92μM-$^{45}CaCl_2$ (sp. radioactivity 9.5Ci/mol) at 37°C and Ca^{2+} accumulation was monitored (●). After 45min the incubation was divided into three portions; all received 197μM-EGTA (brought to pH7.0 with Tris base), plus 2.5mM-Na_2SO_4 (○), 2.5mM-K_2SO_4 (△) or, in the case of the control, 5mM-Tris (brought to pH7.0 with H_2SO_4; ▲). After a further 50min incubation each portion was diluted 10-fold in sucrose medium containing 94μM-$^{45}CaCl_2$ at the same specific radioactivity as before.

inside the ghosts is equal to the concentration outside.

A curved plot was obtained; Ca^{2+}-binding sites were not saturated at 200μM-Ca^{2+}, the highest concentration used. The membranes clearly had high-affinity internal binding sites with K_d in the range 1–10μM as well as sites of lower affinity. No attempt was made to analyse the curve in terms of different binding sites, since the free Ca^{2+} concentration is not known accurately when only low concentrations are used.

Exchange of $^{45}Ca^{2+}$ with Ca^{2+} bound to these sites is presumed to be the mechanism of the observed uptake from solution.

Ca^{2+}/Na^{+} exchange

Ghosts were incubated with $^{45}Ca^{2+}$ as in Fig. 1. If EGTA (Tris salt) is added to the incubation to remove the external free Ca^{2+}, there is an efflux of $^{45}Ca^{2+}$ from the ghosts (Fig. 5). The mechanism of this is uncertain, since we are now no longer observing a Ca^{2+}-exchange phenomenon. If $^{45}Ca^{2+}$ is restored to the incubation medium after depletion of the ghosts, uptake occurs again, although at a slower rate than the initial exchange reaction (Fig. 5, control curve).

To investigate the mechanism of this depletion and re-uptake, which can be repeated through a number of cycles, 5mM-Na^+ or -K^+ was added at the same time as EGTA. It can be seen that Na^+ greatly increased the rate of efflux, whereas K^+ slightly decreased it. On restoration of $^{45}Ca^{2+}$ to the medium to a concentration approximately equal to that in the first (loading) phase of the experiment, ghosts that had been depleted in the presence of Na^+ incorporated Ca^{2+} more rapidly than their K^+- or Tris-treated counterparts.

Re-uptake of $^{45}Ca^{2+}$ cannot now occur by Ca^{2+} exchange, since, after EGTA treatment, the ghosts are largely depleted of internal Ca^{2+}. This experiment suggested that the re-uptake was due to exchange of Ca^{2+} for Na^+, the latter having entered the ghosts by exchange for the internal Ca^{2+} during the depletion with EGTA.

Na^+/Ca^{2+} exchange can be demonstrated directly by adding Na^+ to the incubation medium in the absence of EGTA. This causes a redistribution of Ca^{2+}, with leakage from the ghosts. Examples of this are shown in Figs. 6 and 8, and these are discussed

below. This efflux accounts for the inhibitory effect of Na^+ on Ca^{2+} uptake that was mentioned above.

La^{3+}-induced Ca^{2+} efflux

Addition of La^{3+} to ghosts pre-loaded with $^{45}Ca^{2+}$ for 60 min also induces Ca^{2+} efflux (Fig. 6). We know, from the experiment shown in Fig. 1, that after 60 min incubation the Ca^{2+}-efflux rate from the ghosts is high (in Fig. 1 it is only slightly less than the uptake rate at 60 min, which is 23 nmol/min per mg). La^{3+} inhibits uptake with a K_i of 7 μM; therefore, treatment with 10 μM- and 30 μM-La^{3+}, as used in the experiment shown in Fig. 6, should result in a high Ca^{2+}-efflux rate if Ca^{2+} efflux occurs by a process independent of Ca^{2+} uptake. In fact, the initial efflux rate for both La^{3+} concentrations is about 2.6 nmol/min per mg. This suggests that there is an exchange carrier, catalysing both influx and efflux of Ca^{2+}, the turnover of which is inhibited by La^{3+}.

If both processes are inhibited equally, one would expect no effect of La^{3+} on the steady-state Ca^{2+} concentration. The slow decline actually observed may be due to a slow uptake of La^{3+}, displacing Ca^{2+} from internal binding sites.

Also shown in Fig. 6 is the effect of La^{3+} on Na^+-induced Ca^{2+} efflux. Na^+ alone induces a rapid efflux; La^{3+} decreases the rate of this, although the final equilibrium value reached is unaffected. This is consistent with the idea of Na^+/Ca^{2+} exchange being catalysed by the same protein as Ca^{2+} exchange.

Ruthenium Red was also found to strongly inhibit the leakage of Ca^{2+} from pre-loaded ghosts induced by Na^+ or EGTA. However, since it induced some efflux on its own, it was not possible to investigate its effect in detail.

Sodium fluxes

The coupling of Ca^{2+} movements to Na^+ suggested that it should be possible to monitor Na^+ movements directly. Indeed, incubation of ghosts in solutions containing $^{22}NaCl$ leads to accumulation of $^{22}Na^+$ in a saturable process; in the experiment shown in Fig. 7, time courses showed an uptake of $^{22}Na^+$, reaching a plateau after about 40 min at 37°C. A plot of s/v against s for the data in Fig. 7 gives a K_m for Na^+ of 7.6 mM with a $V_{max.}$ of 65 nmol/min per mg. The mechanism of uptake is not certain; although the ghosts are prepared in sucrose that is buffered by Tris/Hepes, the uptake may occur by exchange of $^{22}Na^+$ with residual Na^+ within the ghosts, or with Ca^{2+}. EGTA was included in the incubation to deplete the ghosts of Ca^{2+}.

As in the case of Ca^{2+} uptake, more than 70% of the accumulated Na^+ is released from the ghosts by subjecting them to osmotic shock. That the $^{22}Na^+$ really is accumulated within ghosts rather than other membrane fragments is shown by sucrose-gradient centrifugation (Fig. 2*c*), when $^{22}Na^+$ co-migrates with $^{45}Ca^{2+}$ and [^{3}H]5-hydroxytryptamine, and

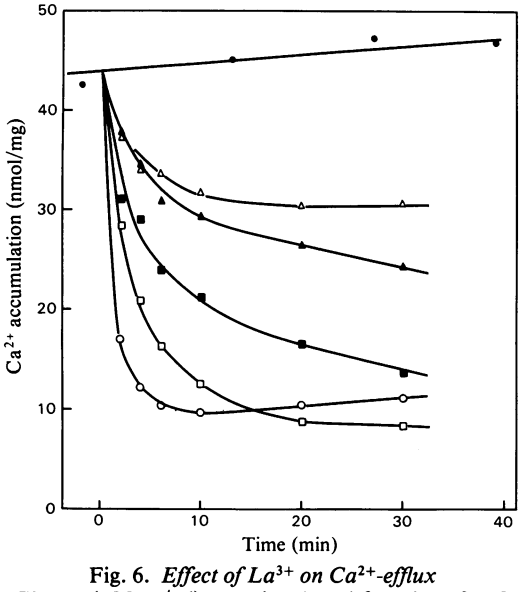

Fig. 6. *Effect of La^{3+} on Ca^{2+}-efflux*
Ghosts (185 μg/ml) were incubated for 1 h at 37°C with 100 μM-$^{45}CaCl_2$ (16 Ci/mol) and Ca^{2+} accumulation was monitored (●). Efflux was induced by adding 10 μM- (△) or 30 μM-$LaCl_3$ (▲); alternatively 10 mM-Na_2SO_4 was added in the absence of La^{3+} (○) or in the presence of 10 μM- (□) or 30 μM-$LaCl_3$ (■).

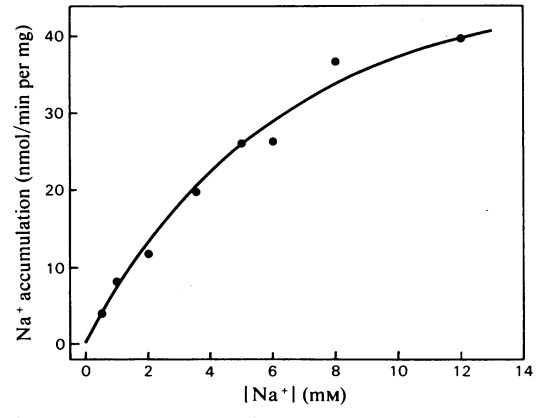

Fig. 7. *Accumulation of Na^+ by chromaffin-granule ghosts*
Ghosts (150 μg/ml) were incubated at 37°C in buffered sucrose containing 1 mM-EGTA, $^{22}NaCl$ (1.9 μCi/ml) and Na_2SO_4 to give the Na^+ concentration shown. Initial rates were assessed over 2 min.

bands at a slightly lower density than the plasma-membrane contaminant, which is revealed by its acetylcholinesterase activity.

The effect of Ca^{2+} on Na^{+} uptake into EGTA-treated ghosts was investigated. Ca^{2+} in the medium had little effect on initial rates of Na^{+} uptake, but the plateau levels of Na^{+} incorporation were decreased when Ca^{2+} was present. The maximum decrease (to about 50% of control levels) occurred when the concentration of Ca^{2+} was over 100μM, half-maximal effects being exerted by 20–30μM-Ca^{2+}. The fact that only part of ghost uptake of $^{22}Na^{+}$ was Ca^{2+}-sensitive, and that Ca^{2+} had rather little effect on initial rates, suggest that the Na^{+}/Ca^{2+} exchanger may not be the only mechanism by which $^{22}Na^{+}$ can enter the ghosts.

If ghosts are incubated with $^{22}Na^{+}$ and Ca^{2+} is subsequently added to the medium, $^{22}Na^{+}$ efflux is induced. Similarly, Na^{+} addition induces Ca^{2+} efflux from ghosts pre-loaded with $^{45}Ca^{2+}$. An example of such an experiment is shown in Fig. 8. Ghosts were incubated with 140μM-Ca^{2+} for 90min at 37°C, leading to equilibration of internal Ca^{2+} with that in the medium. Addition of Na^{+} to the medium leads to an immediate efflux of $^{45}Ca^{2+}$, a new steady-state value being reached within about 10min. The equilibrium position reached presumably reflects an adjustment of internal free Ca^{2+} concentration in response to the change in external Na^{+}. There is a concomitant uptake of Na^{+} (Fig. 8*b*): it can be seen that mole for mole this is slightly faster than the Ca^{2+} efflux, but it takes longer to reach its equilibrium concentration.

It is not possible to solve an equation relating the final Na^{+} and Ca^{2+} values in the experiment in Fig. 8, since the concentrations of the free ions within the ghosts are not known. The experiment was repeated, however, at a lower temperature (25°C) with samples taken at 15s intervals to investigate initial rates. It was found that the initial stoichiometry of Na^{+} influx/Ca^{2+} efflux was between 1 and 2 (1.7 ± 0.5, $n = 8$) nmol of Na^{+}/nmol of Ca^{2+} lost when uptake and efflux values were taken at 2min, with Na^{+} between 1mM and 15mM. Plateau values reached in the experiments corresponded to an uptake of 3.5 ± 0.5nmol of Na^{+}/nmol of Ca^{2+} lost. Initial Ca^{2+} efflux rates showed a hyperbolic dependence on Na^{+} concentration (Fig. 9), with a K_m for Na^{+} of about 5mM and $V_{max.}$ 26nmol/min per mg at 25°C; the efflux rates decreased very rapidly, however, possibly reflecting the heterogeneity of the Ca^{2+} pool within the ghosts, so that the value obtained for $V_{max.}$ may be an underestimate.

Addition of K^{+} to $^{45}Ca^{2+}$-loaded ghosts also led to Ca^{2+} release, but this was considerably decreased

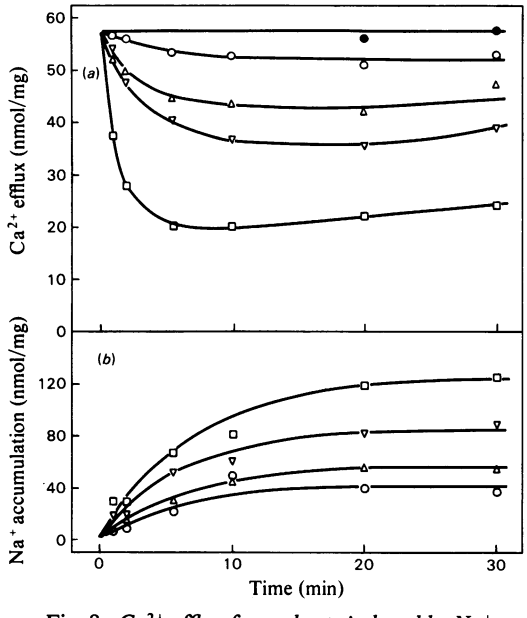

Fig. 8. *Ca^{2+} efflux from ghosts induced by Na^{+}*
Ghosts (160μg/ml) were incubated for 90min at 37°C in two parallel incubations. In (*a*) the medium contained 140μM-$^{45}CaCl_2$ (16Ci/mol) and uptake of $^{45}Ca^{2+}$ was monitored (●). Efflux of Ca^{2+} was initiated by addition of Na_2SO_4 to give 2.2 (○), 4.7 (△), 7.9 (▽) or 15.6 (□) mM-Na^{+}. In (*b*) the uptake medium contained non-radioactive 140μM-$CaCl_2$. Efflux of Ca^{2+} was initiated as in (*a*), but media contained $^{22}NaCl$ (1.6μCi/ml) and uptake of Na^{+} was monitored. Symbols are as defined in (*a*).

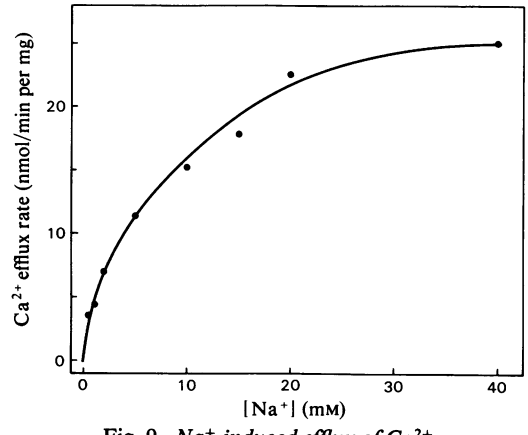

Fig. 9. *Na^{+}-induced efflux of Ca^{2+}*
Ghosts (190μg/ml) were incubated for 130min at 25°C in buffered sucrose medium containing 150μM-$^{45}CaCl_2$ (sp. radioactivity 13Ci/mol). Na_2SO_4 was added to give the concentrations shown and $^{45}Ca^{2+}$ efflux was monitored by filtering samples at 15s intervals.

compared with the effect of Na^+. K^+ does not inhibit the Na^+-induced efflux.

Is a membrane potential developed during Na^+/Ca^{2+} exchange?

Rates of Ca^{2+} uptake or efflux should be unaffected by a membrane potential if catalysed by a Ca^{2+}/2 Na^+ antiporter, but should be sensitive to potential if another stoichiometry is involved. Prolonged membrane potentials (positive inside) can be imposed across ghost membranes by hydrolysis of ATP in the medium catalysed by the H^+-translocating ATPase of the membrane (Phillips & Allison, 1978); the potential is dissipated by inclusion of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

$^{45}Ca^{2+}$ efflux from pre-loaded ghosts was induced by addition of 4mM-Na^+ in the presence of 1mM-$MgATP^{2-}$. Efflux rates were identical in the presence and absence of uncoupler. Similarly, $^{45}Ca^{2+}$ uptake was followed into ghosts that had been pre-incubated for 1h in a medium containing EGTA and Na^+; the uptake rate in the presence of 1mM-$MgATP^{2-}$ was unaffected by addition of either 4μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or of 4mM-KI (the latter was compared with a control containing 2mM-K_2SO_4). It therefore seems unlikely that the Na^+/Ca^{2+} exchange is electrogenic.

Ammonium ions, which dissipate the $MgATP^{2-}$-induced pH gradient across the membrane, but enhance the membrane potential, were also tested; however, 4mM-$(NH_4)_2SO_4$ was in fact somewhat inhibitory to $^{45}Ca^{2+}$ uptake, and it was therefore not investigated further.

Discussion

Ca^{2+} entry into chromaffin granules appears to occur by Ca^{2+}/Na^+ exchange. A similar exchange is found in the plasma membrane of excitable cells, and in certain mitochondria (Carafoli, 1979). In the former case, three or more Na^+ ions exchange with each Ca^{2+} [squid axon plasma membrane (Blaustein, 1976); cardiac sarcolemma (Pitts, 1979; Reeves & Sutko, 1980; Caroni *et al.*, 1980; Philipson & Nishimoto, 1980)], providing an electrogenic transport system. In the case of the Ca^{2+}/Na^+ exchanger of heart mitochondria, two or more Na^+ exchange per Ca^{2+} (Crompton *et al.*, 1977). It has been clearly shown that the same carrier catalyses Ca^{2+}/Ca^{2+} exchange; furthermore, it is quite distinct from the well-known mitochondrial Ca^{2+}-uniporter (Panfili *et al.*, 1981).

Chromaffin-granule Ca^{2+} transport does not seem to resemble other Ca^{2+}-transport mechanisms such as (i) that catalysed by Ca^{2+}-stimulated ATPases in sarcoplasmic reticulum or plasma membranes; (ii) ATP-stimulated uptake into vesicles such as cholinergic synaptic vesicles from *Torpedo* (Michaelson *et al.*, 1980), rat brain synaptosomal vesicles (Rahaminoff & Abramovitz, 1978) or neurohypophyseal microvesicles (Nordmann & Chevalier, 1980); or (iii) Ca^{2+}/H^+ exchange, which is probably found in mitochondria from a variety of sources, including liver (Fiskum & Lehninger, 1979).

The chromaffin-granule transport system most resembles that from heart mitochondria, catalysing Ca^{2+} exchange as well as Ca^{2+}/Na^+ exchange. Though its measured K_m is rather higher (38μM compared with 13μM; Crompton *et al.*, 1977), its $V_{max.}$ is of the same order of magnitude. The kinetic parameters given must be regarded with caution, however, as the radioactive quench technique is not good for estimates of initial velocity when these rates are linear for less than 1min, and no attempt was made to buffer very low Ca^{2+} concentrations in view of the relatively high K_m found. Furthermore, erythrocyte Ca^{2+} transport has been shown to be activated by calmodulin (MacIntyre & Green, 1978; Larsen & Vincenzi, 1979) and the effect of this protein on chromaffin-granule transport has not yet been tested.

A similar problem affects determination of the Ca^{2+}/Na^+ stoichiometry. Work on other Ca^{2+}/Na^+ exchangers has shown a marked sigmoidicity in the dependence of the rate of Ca^{2+} flux on Na^+ concentration, but this was not seen in the present work (Fig. 9). Again, however, initial rates were rapid, and are hard to determine accurately. The lack of effect of membrane potential on the flux rates would suggest a stoichiometry of Ca^{2+}/2 Na^+, which would be expected to show sigmoid kinetics.

Na^+ uptake, which has not been investigated in detail, appears to contain two components, only one of which is Ca^{2+}-sensitive. This clearly merits further investigation since, at the moment, the biological role of the Ca^{2+} transport system is unclear. It has been argued that dual Ca^{2+} transport systems in mitochondria function to regulate either cytoplasmic free Ca^{2+}, or intramitochondrial Ca^{2+}, or both (Nicholls & Crompton, 1980; Denton & McCormack, 1980). At present the evidence favours a single Ca^{2+} transport system in chromaffin granules, suggesting that precise regulation of intragranular Ca^{2+} is not possible. This is supported by the work of Serck-Hanssen & Christiansen (1973), who showed that the calcium content of intact chromaffin granules doubled after extensive acetylcholine-induced catecholamine secretion from perfused bovine adrenal glands. The role of Ca^{2+} transport is thus envisaged as being to scavenge Ca^{2+} entering the cell during plasma-membrane depolarization, the granule releasing it from the cell during exocytosis. In this respect the adrenal medulla is seen as contrasting with nerve cells in the

central nervous system; Nicholls & Scott (1980) and Scott *et al.* (1980) have argued that mitochondria in the brain form the main short-term reservoir for Ca^{2+}, the concentration of which is then regulated by the activity of the plasma membrane. The adrenal medulla may be different in view of its large content of secretory granules, and relative lack of mitochondria, in the vicinity of the plasma membrane.

The uptake of Ca^{2+} by chromaffin granules would be dependent on intragranular Na^+, which would require an additional entry mechanism that is independent of Ca^{2+}. At present there is no evidence for ATP-dependent Na^+ uptake (J. H. Phillips, unpublished work), for a ouabain-sensitive ATPase (Apps *et al.*, 1980) or for the Na^+/H^+ exchange (Phillips, 1977) in the chromaffin-granule membrane. However, the uptake of 3–4 Na^+/Ca^{2+} lost when ghosts are exposed to external Na^+ (Fig. 8) and the uptake of Na^+ in the presence of saturating external Ca^{2+} both suggest that an alternative Na^+ entry mechanism exists.

It would clearly be of some interest to extend these studies to intact granules again, as investigated originally by Kostron *et al.* (1977), to define the role of Na^+ in the presence of the intact granule matrix of catecholamine, ATP and protein.

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