BOTH THE TRANSMEMBRANE pH GRADIENT AND THE MEMBRANE POTENTIAL ARE IMPORTANT IN THE ACCUMULATION OF AMINES BY RESEALED CHROMAFFIN-GRANULE 'GHOSTS'

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1. Introduction

The secretory granules of the adrenal medulla, known as chromaffin granules, contain high concentrations of catecholamines and ATP, associated with soluble proteins (reviewed [1]). Catecholamines are transported into the granule matrix by a reserpine-sensitive permease, which shows specificity for the (−) forms of adrenaline and noradrenaline, and also transports dopamine and 5-hydroxytryptamine [2]. Accumulation of these substrates by intact granules is driven by ATP, and it is well established [3–5] that an ATPase in the chromaffin granule membrane translocates protons into the granule; this results in creation of a transmembrane proton gradient (ΔpH) and potential ($\Delta\psi$), amine uptake being dependent upon these, rather than on ATP hydrolysis itself. Details of the mechanism by which amine uptake is coupled to the collapse of ΔpH and/or $\Delta\psi$ have not, however, been elucidated; we now report some studies on the initial rates of uptake of noradrenaline, dopamine and 5-hydroxytryptamine which suggest that transport can be driven by an imposed ΔpH, in the absence of $\Delta\psi$, but that the rate of uptake is increased by superimposition of a membrane potential.

2. Materials and methods

Resealed chromaffin granule 'ghosts' were prepared by a modification of the method in [6]. Freshly prepared bovine chromaffin granules were lysed at 0°C by ~50-fold dilution into 10 mM Hepes–NaOH (pH 7.0); the sucrose concentration was readjusted to 0.3 M, the membranes collected by centrifugation (30 min, 23 000 rev./min, 4°C in a Beckman type 35 rotor, g_{av} = 41 000), resuspended in 0.3 M sucrose to ~2 mg protein/ml, and 5 ml portions layered on discontinuous gradients of 4.5 ml 0.4 M sucrose over 2.5 ml 0.4 M sucrose in ^2H_2O. All solutions were buffered with 10 mM Hepes–NaOH (pH 7.0). The gradients were centrifuged (30 min, 40 000 rev./min, 4°C in a Beckman SW41 rotor, g_{av} = 196 000) and the 'ghosts' collected from the sucrose/sucrose–^2H_2O interface; the final protein conc. was 2.5–4.5 mg/ml, and associated catecholamine ~150 nmol/mg protein. Specific ATP-hydrolytic and 5-hydroxytryptamine uptake activities did not decline significantly on storage of the 'ghosts' for 2 weeks at −20°C; in all experiments reported, 'ghosts' had been frozen and thawed once.

ATP-dependent uptake of ^{14}C-labelled catecholamines or 5-hydroxytryptamine was determined as in [6]. Uptake driven by an imposed ΔpH was determined by a modification of the procedure in [7]: 'ghosts' (~0.8 mg protein/ml) were incubated for 15–30 min at the required temperature in 0.3 M sucrose, 10 mM Mes–NaOH (pH 5.6) together with the radiolabelled substrate (1 μCi/ml; 16–20 μM) and valinomycin (2 μg/ml). Aliquots (50 μl) were removed and diluted with 50 μl 88 mM Hepes–NaOH (pH 8.8) in 0.3 M sucrose, containing ammonium, potassium or sodium salts as required: this produced a final pH 8.0. Uptake was stopped by dilution of each sample with 2.5 ml ice-cold 0.3 M sucrose–

Abbreviations: ATP, adenosine 5'triphosphate; ATPase, adenosine triphosphatase (EC 3.6.1.3); Hepes, *N*-2-hydroxyethyl-*N'*-2-ethane sulphonic acid; Mes, 2-*N*-morpholinoethane sulphonic acid

10 mM Hepes–NaOH (pH 7.0) and rapid filtration through cellulose nitrate filters (Sartorius, 2.5 cm diam., 0.45 μm pore size). The filters were dried and counted for radioactivity in toluene-based scintillator. Uptake curves were extrapolated to zero time in each case.

Uptake driven by an imposed potential was measured in a similar way: 'ghosts' (0.8 mg protein/ml) were preincubated in 0.3 M sucrose, 20 mM Hepes–NaOH (pH 7.0) together with the radiolabelled substrate (0.5 μCi/ml; 26–30 μM) and valinomycin (1.3 μg/ml); 100 μl aliquots removed, rapidly mixed with 10 μl 0.3 M sucrose (for blank determinations) or 10 μl 0.5 M K_2SO_4, then treated as above. Background counts, obtained with preincubated ghosts lysed with 10 mM Hepes and filtered, were subtracted.

The sizes of the proton gradient (ΔpH) or membrane potential ($\Delta\psi$) generated in these experiments were determined by the [^{14}C]methylamine and $^{14}CNS^-$ distribution methods, respectively [7,8]. Samples of ghosts (50–100 μg protein) were filtered without dilution on 13 mm diam. cellulose nitrate filters and the filters counted for radioactivity without washing or drying, in scintillator containing 33% (v/v) Triton X-100.

Valinomycin and nigericin were from Eli Lilly, Indianapolis; (±) [^{14}C]noradrenaline, [^{14}C]dopamine, 5-[^{14}C]hydroxytryptamine, K^{14}CNS and [^{14}C]methylamine from the Radiochemical Centre, Amersham; Hepes, Mes, reserpine ATP and unlabelled substrates were from Sigma.

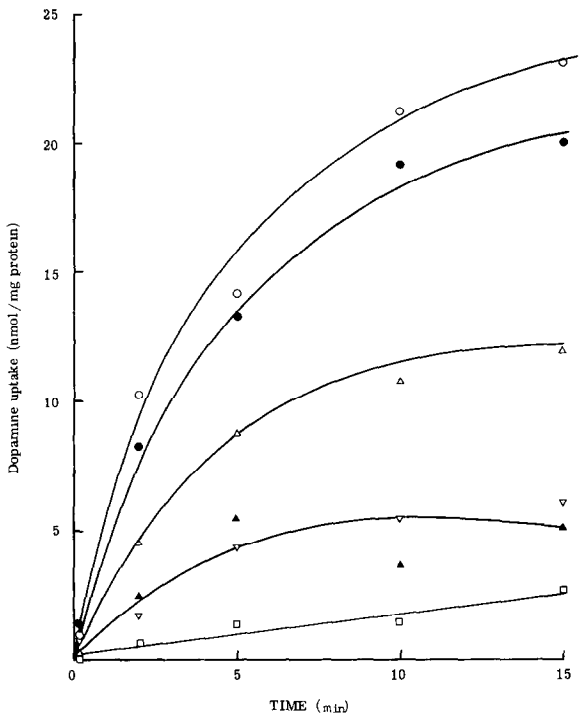


Fig.1. ATP-dependent uptake of dopamine at 37°C. The incubation mixture contained 'ghosts' (0.4 mg protein/ml), 0.3 M sucrose, 20 mM Hepes–NaOH (pH 7.0), 6 mM ATP, 3 mM $MgSO_4$, 59 μM dopamine (8.5 Ci/mol) and the following additions: (○) none; (●) 10 mM KCNS; (△) 50 ng nigericin/ml; (▲) 50 ng/ml nigericin, 10 mM K_2SO_4; (▽) 10 mM $(NH_4)_2SO_4$; (□) 8.3 μM reserpine.

3. Results and discussion

3.1. *ATP-driven amine uptake*

Accumulation of dopamine by resealed chromaffin-granule 'ghosts', at 37°C in the presence of $ATPMg^{2-}$, is shown in fig.1. Results obtained with (−)noradrenaline were qualitatively similar (not shown), while uptake of 5-hydroxytryptamine was less sensitive to reserpine, and showed some stimulation by thiocyanate. Initial rates of uptake of these 3 substrates, under the various conditions, are given in table 1, together with the values of ΔpH and $\Delta\psi$ in each case.

3.2. *ΔpH-driven uptake*

Our aim in these experiments was to investigate the effect of a superimposed membrane potential on the reported [7] uptake of amines driven by an imposed ΔpH. The experiments were performed at a lower temperature than used in [7] as we have found that the potential induced by a potassium concentration gradient decays rather rapidly at 37°C (unpublished; see [11]). Fig.2 shows uptake of 5-hydroxytryptamine at 25°C, under conditions in which both ΔpH and $\Delta\psi$ were induced (addition of K_2SO_4), ΔpH only (addition of KSCN, or Na_2SO_4) or $\Delta\psi$ only (addition of K_2SO_4, in presence of low concentrations of NH_4^+). Similar experiments with (−)noradrenaline and dopamine, performed at 30°C because of the low rates of uptake of these substrates at 25°C, yielded qualitatively similar results.

3.3. *Δψ-driven uptake*

We attempted to measure amine uptake driven by a potassium diffusion potential at pH 7.0, in the

Table 1
Initial rates of amine uptake by chromaffin granule 'ghosts' (nmol . mg^{-1}. min^{-1})

	Dopamine (59 μM)	5-Hydroxytryptamine (59 μM)	(−)Noradrenaline (55 μM)[a]	ΔpH	Δψ (mV)
No addition	6.7	8.8	3.9	0.81	72
KCNS (10 mM)	4.7	11.1	3.6	1.21	–
Nigericin (50 ng/ml)	3.6	7.5	2.6	0.65	75
Nigericin (50 ng/ml) K_2SO_4 (10 mM)	1.1	4.0	1.5	0.56	78
$(NH_4)_2SO_4$ (10 mM)	1.0	1.1	0.8	0.0	80
Reserpine (8.3 μM)	0.2	0.9	0.1	1.02	58

[a] This reaction mixture contained 10 μM (±) [^{14}C]noradrenaline (52 Ci/mol) with 50 μM (−)noradrenaline as carrier

The conditions used are given in the legend to fig.1

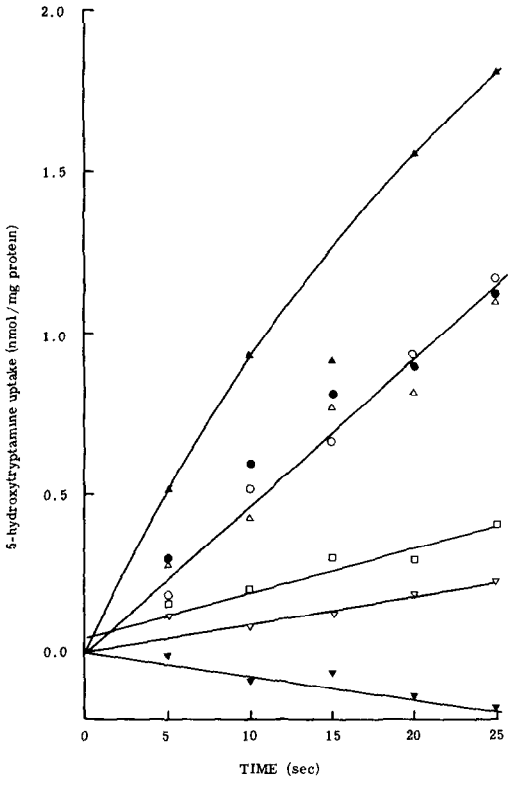


Fig.2. ΔpH-driven uptake of 5-hydroxytryptamine at 25°C. 'Ghosts' were preincubated at pH 5.3 with 17.2 μM 5-hydroxytryptamine (58 Ci/mol) and 2 μg/ml valinomycin, and transferred to an equal volume of medium at pH 8.8, containing the following additions: (○) none; (▲) 50 mM K_2SO_4; (△) 50 mM Na_2SO_4; (●) 100 mM KCNS; (□) 10 μM reserpine; (▽) 50 mM K_2SO_4, 10 μM reserpine; (▼) 50 mM K_2SO_4, 10 mM $(NH_4)_2SO_4$.

absence of imposed ΔpH: the results for 5-hydroxytryptamine, at 30°C, are shown in fig.3. Similar results were obtained with (−)noradrenaline.

3.4. *The mechanism of catecholamine uptake*

The simplest model of amine uptake predicts that these substrates are accumulated in the same way as methylamine, the low pH inside the vesicle producing an increase in the internal concentration of protonated

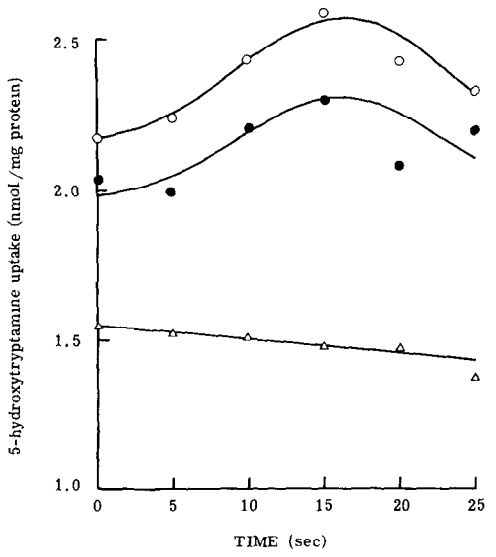
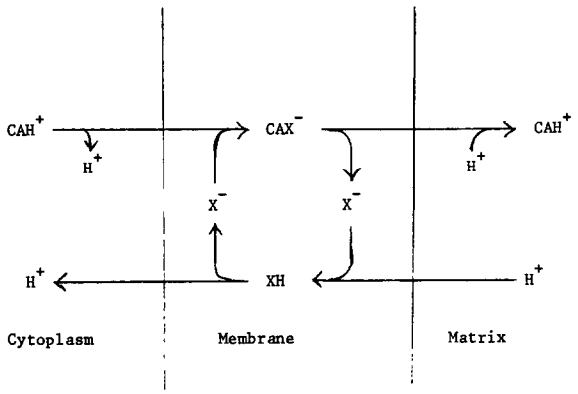


Fig.3. Δψ-driven uptake of 5-hydroxytryptamine at 30°C. 'Ghosts' were preincubated with 29 μM 5-hydroxytryptamine (17.4 Ci/mol) and 1.2 μg/ml valinomycin, and the potential generated by the addition of 45 mM K_2SO_4, with the following additions: (○) none; (●) 2.5 mM CH_3COONa; (△) 10 μM reserpine.

substrate. This effect has been demonstrated in liposomes [12], although in chromaffin granules entry of catecholamines is presumably via a permease. The sum of the two processes, electrogenic ATP-driven H^+-translocation and electroneutral catecholamine uptake, would be electrogenic, leading to accumulation of protonated catecholamine, and producing a membrane potential greater than is actually measured [7]; some mechanism of neutralizing this potential, such as anion uptake or cation efflux, must therefore operate. A further objection to this simple model is that chromaffin granules, in the absence of ATP, show a transmembrane ΔpH of ~1.5, but do not accumulate catecholamines at a significant rate; on addition of $ATPMg^{2-}$, catecholamine uptake is stimulated, although ΔpH is hardly altered, whilst $\Delta\psi$ is greatly increased [4,8]. In [8], a model was proposed which elegantly accounts for this observation: this is shown in scheme 1, in which X is the catecholamine permease, and CA is catecholamine.

The sum of these processes is exchange of CAH^+ of 2 H^+, catecholamine uptake resulting in alkalinization of the vesicle interior and generation of a potential, negative inside. The model predicts that uptake can be driven by ΔpH (acid inside), providing that the developing potential can be dissipated by other ion movements; and since transport is electrogenic, $\Delta\psi$ (positive inside) would promote uptake. Our results are largely in support of this scheme: ATP-driven uptake is very sensitive to reduction of ΔpH by NH_4^+ or K^+ plus nigericin (fig.1, table 1). Nigericin alone also reduces ΔpH, presumably by catalysing exchange of H^+ with Na^+, the counter-ion to the Hepes buffer: it therefore inhibits uptake.



Scheme 1

A qualitative difference between the effects of CNS^- on the uptake of 5-hydroxytryptamine and of dopamine and noradrenaline is apparent (table 1); this may be because the reserpine-insensitive component of uptake, which may arise through uncatalysed uptake of the unprotonated amine in response to the H^+-gradient, is large for 5-hydroxytryptamine, but not for the other amines. The permeant anion CNS^- tends to collapse $\Delta\psi$ whilst increasing ΔpH: in keeping with scheme 1, this is inhibitory to uptake of dopamine and noradrenaline, but the increase in ΔpH and decrease in $\Delta\psi$ would both favour uncatalysed accumulation of 5-hydroxytryptamine, which may obscure the effects on permease-mediated uptake. This interpretation is supported by the observation that at 25°C 5-hydroxytryptamine uptake is more sensitive to reserpine (96% inhibition, compared to 90% at 37°C) and stimulation by CNS^- is no longer observed.

In experiments in which a transmembrane ΔpH is imposed in the absence of ATP (fig.2) the rate of amine uptake is approximately doubled, from 2.7 to 6.2 nmol . mg protein^{-1}. min^{-1}, by superimposition of a potential with 50 mM K_2SO_4 plus valinomycin; 100 mM Na^+, or K^+ in the presence of CNS^-, fail to have any stimulatory effect. This rate of substrate uptake is surprisingly high, considering the low temperature and substrate concentration; but the ΔpH measured in pH-jumps is ~2.1, which is considerably larger than that found with this 'ghost' preparation in the presence of ATP.

The potential induced by K^+-valinomycin in these experiments is 41 mV (95% confidence limits, 32–48 mV). If K^+ is replaced by Na^+, or if valinomycin or K^+ are omitted, we are unable to measure any potential; presumably acidification of the vesicle during the low-pH preincubation occurs by electroneutral Na^+/H^+ exchange. The measured ΔpH is unaffected by the addition of K^+ or Na^+, and decays during the course of uptake, the methylamine concentration ratio being halved in ~80 s.

In the presence of NH_4^+, uptake of NH_3 collapses the applied ΔpH, and totally inhibits uptake (fig.2). ATP-dependent uptake is not completely inhibited by NH_4^+, however (table 1); under these conditions, creation of ΔpH is electrogenic, and its collapse by NH_3 uptake results in an increase in $\Delta\psi$. This suggests that $\Delta\psi$ alone may be able to drive uptake, and we attempted to measure potential-driven uptake in the absence of ΔpH using a K^+ diffusion potential at

pH 7.0 (fig.3). The size of this potential, measured by CNS^- distribution, was 57 mV (95% confidence limits, 66–43 mV). The uptake induced by this potential is transient, and small relative to the amount of bound substrate which can be removed by hypotonic lysis; but it is sensitive to reserpine. Potential-driven uptake of adrenaline has been reported [11], although the kinetics of uptake observed in [11] were different, perhaps because the 'ghosts' were prepared in a different way, and, in particular, contained a different internal buffer. A further difference in our results is the lowered levels of membrane-associated substrate found after incubation with reserpine (fig.3). This may be a result of inhibition of substrate binding by the inhibitor, or of reduction of passive uptake during preincubation.

According to scheme 1, potential-driven substrate uptake would be opposed, and ultimately limited, by internal alkalinization of the vesicles (although the internal buffering capacity should be sufficient to overcome the small pH-changes expected in our experiments). We investigated the effect of low concentrations of acetate, which should neutralize this ΔpH by inward movement of acetic acid, but were unable to detect any stimulation (fig.3).

In conclusion, we believe that our results support the model of Johnson and Scarpa [8], since they suggest that amine uptake is stimulated by an electrical potential in the presence of ΔpH, and is therefore electrogenic; however the prediction that $\Delta\psi$ alone can drive uptake is only marginally fulfilled under the conditions of our experiments.

Results obtained with resealed 'ghosts' in [13] are consistent with their model of uptake and the observations reported here, although the 'ghosts' in [13], prepared by a different method, show very much lower rates of amine uptake than those used in our experiments.

Acknowledgement

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