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ROLE OF THE PROTON ELECTROCHEMICAL GRADIENT IN MONOAMINE TRANSPORT BY BOVINE CHROMAFFIN GRANULES

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Summary

The role of the transmembrane potential ($\Delta\psi$), the proton concentration gradient (ΔpH) and the proton electrochemical gradient ($\Delta\mu_{H^+}$) in monoamine uptake by bovine chromaffin granules or ghosts was investigated. In presence of ATP the permeant anion SCN^- collapsed the $\Delta\psi$ (inside positive) and inhibited monoamine uptake by granules or well buffered ghosts. With lightly buffered ghosts, SCN^- induced an acidification which resulted in a low inhibition of uptake. Cation efflux as well as anion influx affected the $\Delta\psi$, and a transient valinomycin-mediated K^+ efflux induced a lag in the uptake. The ΔpH-driven noradrenalin uptake was also sensitive to $\Delta\psi$, since superimposing a positive or a negative $\Delta\psi$ to the ΔpH, respectively, increased or decreased the rate of noradrenalin accumulation. A ΔpH was required for this increase of uptake rate, which was proportional to the $\Delta\psi$. The pH-dependence of the ATP-induced monoamine uptake by granules pointed to the $\Delta\mu_{H^+}$ as the driving force. In contrast with the rate of uptake, which was not dependent on the anions present, the extent of amine incorporation was decreased when the internal anionic buffer concentration was decreased and, at a low internal buffer concentration, when ATP anion transport was blocked.

Introduction

In presence of ATP, chromaffin granules, the catecholamine storage organelles of adrenal medulla, and 'ghosts' derived from these organelles, have been

Abbreviations: OX-V, bis(3-phenyl-5-oxoisoxazol-4-yl) penthamethine oxonol; Hepes, *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulfonic acid; PPO, 2,5-biphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-1-benzene; ANS, 8-anilinonaphthalene 1-sulphonate.

shown to accumulate catecholamines against their concentration gradient [1,2]. The Mg^{2+}-activated ATPase of the granule membrane is involved in this process [3]. This enzyme has a H^+-translocase activity and may generate a pH gradient (ΔpH, inside acidic) [4,6] and/or a transmembrane potential ($\Delta\psi$, inside positive) [5,7—10]. Despite a multiplicity of evidence pointing to a chemiosmotic mechanism, the nature of the coupling between monoamine uptake and proton pump has not yet been elucidated: is the driving force arising from the ΔpH, the $\Delta\psi$ or the proton electrochemical gradient ($\Delta\mu_{H^+} = \Delta\psi + 60\ \Delta pH$ at 37°C)?

Amine uptake by ghosts [11,12] or granules [13] to which a ΔpH (inside acidic) is imposed suggests that a ΔpH generated by the H^+-pump is the driving force of the ATP-induced uptake [14,15]. However, we have shown that methylamine, which is transported through the membrane according to the pH gradient, is accumulated much less than noradrenalin when ghosts are energized by addition of ATP [30]. On the other hand, convincing evidence of the role of the $\Delta\psi$ in the ATP-induced uptake has been recently given [9,10]. Njus and Radda [16] and later Johnson and Scarpa [10] have proposed electrically dissipative models for the mononamine uptake which involve a carrier-mediated proton antiport, the exergonic requirements of the active transport being fulfilled by the electrochemical potential decrease of the exchanged proton.

The present report confirms the contribution of the $\Delta\psi$ to catecholamine uptake and supports electrodissipative models. We show that an externally imposed $\Delta\psi$ affects the ΔpH-induced noradrenalin uptake. Our data point to the $\Delta\mu_{H^+}$ as the driving force of monoamine uptake. In addition, the role of anions, which is important in models involving a ΔpH as the driving force for accumulation, has been investigated and found to be consistent with the electrodissipative model. 'Ghosts' derived from chromaffin granules have been used throughout this study with the exception of experiments where a well buffered internal medium was required, in which case granules were used [20]. In several cases, the dye OX-V has been used to monitor $\Delta\psi$, since changes of inside positive transmembrane potentials are proportional to the dye relative fluorescence changes [31].

During completion of the present work, it has been shown independently by Njus and Radda that a K^+ diffusion potential caused adrenaline uptake in chromaffin granule ghosts [17]. A preliminary account of some of the experiments reported in this publication has already been published [18].

Materials and Methods

Materials

[*side chain*-2-^{14}C]Tyramine hydrochloride (50 mCi/mmol), (—)-[7,8-^{3}H]noradrenalin acetate (15 Ci/mmol), [^{14}C]thiocyanate (59 mCi/mmol) and D-[U-^{14}C]sorbitol (191 mCi/mmol) were obtained from the Radiochemical Center (Amersham, U.K.); [^{14}C]methylamine (38 mCi/mmol) and ^3H_2O were from CEA, France. OX-V and tetrabenazine were gifts of Dr. B.S. Cooperman and Hoffman-La Roche (Basle, Switzerland), respectively. Valinomycin (4 mM), OX-V (0.2 mM) and tetrabenazine (4 mM) were dissolved in ethanol, atractyloside (20 mM) in ethanol/H_2O (1 : 1).

Granule and ghost preparation

Chromaffin granules were prepared by differential centrifugation in 0.3 M sucrose/10 mM NaOH/Hepes buffer, pH 7.0 [8]. To prepare ghosts, the granules (15—20 mg protein/ml) were lysed by dropwise addition to 20 vols. of an hypo-osmotic solution containing 2 mM $MgSO_4$, 0.1 mM dithiothreitol, 10 μM $CaCl_2$, and K_2SO_4 and buffer as indicated [15]. The suspension was stirred for 10 min at 4°C and was centrifuged at 37 000 × *g* for 20 min. The pellet was washed under the same conditions and was resuspended at a concentration of 10—15 mg protein/ml in 0.3 M sucrose, 2 mM $MgSO_4$, 0.1 mM dithiothreitol and K_2SO_4 and buffer as in the lysis solution.

Monoamine uptake

The composition of the various incubation mixtures is given in the legend of the figures. When valinomycin, tetrabenazine or atractyloside were added to the incubation the granule or ghost preparation was pretreated for 4 min at 4°C with the same drug concentration and controls were performed with the same ethanol concentration (less than 1%). The final radioactivity of the amines was, per ml: (—)-[7,8-^{3}H]noradrenaline, 4 μCi; [*side chain*-2-^{14}C]tyramine, 2 μCi and [^{14}C]methylamine, 0.38 μCi. Incubations were initiated by addition of the membrane preparation. Aliquots (100 μl) were withdrawn at intervals, diluted in ice-cold incubation buffer (1 ml) and filtered on Millipore filters (HAWP). The filters were washed twice with the same buffer (2 ml), dried and counted in toluene containing PPO (5 g/l) and POPOP (0.3 g/l). Results were corrected for amine adsorption on the filter (generally less than 10% of the uptake). When uptake rates were measured, time courses were determined and the initial rates obtained in the linear part of the reaction were used.

OX-V fluorescence measurements

OX-V fluorescence changes were measured as described [31] with a Jobin-Yvon double monochromator fluorimeter, using 2-ml cuvettes. Excitation and emission wavelengths were 580 and 650 nm, respectively. Data were expressed as relative fluorescence decrease.

Transmembrane potential and pH gradient measurements

1. KS^{14}CN and [^{14}C]methylamine partition technique. The procedure of Casey et al. [4] was applied. For each measurement, four 0.25-ml aliquots of chromaffin granules (3 mg of protein) were mixed with the same volume of 0.3 M sucrose/50 mM Tris-succinate (at various pH)/5 mM ATP/2.5 mM $MgSO_4$. After a 3-min incubation at 20°C, isotopes (20 μl) were added: ^3H_2O and D-[U-^{14}C]sorbitol to two samples for determination of the internal exchangeable water space (sorbitol excluding volume) and to the two remaining samples, ^3H_2O and S^{14}CN for positive $\Delta\psi$ or ^3H_2O and [^{14}C]methylamine for ΔpH determination. Final isotope activities were (in μCi/ml): ^3H_2O, 2; D-[U-^{14}C]-sorbitol 0.1 mM, 1; S$^{14}CN^-$ 25 μM, 1.5; [^{14}C]methylamine 36 μM, 1.35. The samples were equilibrated for 3 min at 20°C, centrifuged for 10 min at 27 000 × *g* at the same temperature and the pellets were dissolved in 0.5 ml of 2% Triton X-100. Pellet and supernatant aliquots were counted in a Triton/toluene scintillation fluid. Internal water space, and internal to external concen-

tration ratios of SCN^- and methylamine were derived as in Ref. 4 from the relative activities of the isotopes in pellets and supernatants. Granule internal water space was 2.5—2.6 μl/mg of protein. $\Delta\psi$ and ΔpH were calculated as:

$$\psi_{in} - \psi_{out}(mV) = 57.6 \log \frac{(SCN)_{in}}{(SCN)_{out}} \quad \text{at } 22°C$$

$$pH_{out} - pH_{in} = \log \frac{(MeNH_2)_{in}}{(MeNH_2)_{out}}$$

2. Determination of K^+-diffusion potential. The internal K^+ concentration was determined to allow calculation of the imposed K^+ diffusion potential by the Nernst equation. It was obtained as the null point of K^+ diffusion potentials monitored by the OX-V effect and resulting from suspension of aliquots of the sample in media containing valinomycin and various K^+ concentrations. Ghosts were incubated at a concentration of about 0.1 mg protein/ml in 0.3 M sucrose, 5 mM NaOH/Hepes (pH 7.0) and various concentrations of K_2SO_4, at 25°C. The addition of valinomycin (10 μM) induced a slow decrease of the dye fluorescence (excitation 580 nm, emission 650 nm) which monitored the $\Delta\psi$ [31]. The relative fluorescence change was plotted as a function of the log of the external K^+ concentration and the internal K^+ concentration was derived from the intersect with the abscissa of the straight line thus obtained (correlation coefficient 0.997, 0.994 and 0.980 in separate experiments). These values were higher than the K^+ concentration of the lysis buffer (about 5-times), indicating a shrinkage of the ghosts during their resuspension.

3. Determination of ghosts' internal pH. Internal pH were derived from measurements of the ΔpH-driven methylamine uptake at various pH. Ghosts (about 1 mg protein/ml) were incubated at 25°C in 0.3 M sucrose/25 mM Tris-succinate at various pH and 10 μM [^{14}C]methylamine. Aliquots in triplicate were rapidly withdrawn and their radioactivity measured as described. To perform a control at $\Delta pH = 0$ the samples were preincubated for 30 min at 37°C in a pH 7.0 buffer before addition of [^{14}C]methylamine. The log of the [^{14}C]methylamine concentration was plotted as a function of the external pH and the intersect of the straight line thus obtained (correlation coefficient 0.995) with the control at $\Delta pH = 0$ was taken as the internal pH. This value was generally 0.5 pH unit more acidic than the medium, which might be attributed to a Donnan effect [5].

ATP-induced noradrenalin uptake at low anion concentration

In addition to ghosts (2.0 mg protein/ml) and 0.3 M sucrose/20 mM Hepes buffer (pH 7.0), the incubation contained 0.2 mM atractyloside, 5 mM ATP neutralized by 2.5 mM $Mg(OH)_2$ and NaOH, and 100 μM noradrenalin associated with various anions. The catecholamine salts were prepared by exchange of the tartrate on Dowex AG 2-X8 columns equilibrated with the desired anion. Rates of uptake were measured at 37°C and were compared to a control (2.6 nmol/mg protein per min) incubated in the absence of atractyloside and in the presence of $MgSO_4$ (2.5 mM) and 100 μM noradrenalin chloride.

ATPase activity

Ghosts (50—100 μg protein/ml) were incubated at 37°C with ATP, sodium

salt (2 mM) and $MgSO_4$ (2 mM) in 25 mM NaOH/Hepes or Tris-succinate buffer at the indicated pH. Oligomycin (10 μg/ml) was added to the incubation mixture to inhibit the mitochondrial ATPase. Aliquots (50 μl) were withdrawn at 5 and 10 min and assayed for inorganic phosphate [19].

Protein assay

Proteins were estimated by the method of Lowry et al. [32] with bovine serum albumin as a standard, following precipitation in 5% trichloroacetic acid and redissolution in 2% deoxycholate/3% NaOH.

Results

Effect of KSCN on granule and ghost energization and monoamine uptake

The permeant anion SCN^- is known to affect the positive transmembrane potential generated by the proton pump of chromaffin granules or ghosts [9–11]. However, if the inhibition of the $\Delta\psi$ (monitored by the OX-V effect) were similar for the two preparations (Fig. 1A and B), thiocyanate affected in a different way monoamine uptake by granules (Fig. 1A) and by ghosts (Fig. 1B). With granules, noradrenalin uptake was efficiently blocked by KSCN and

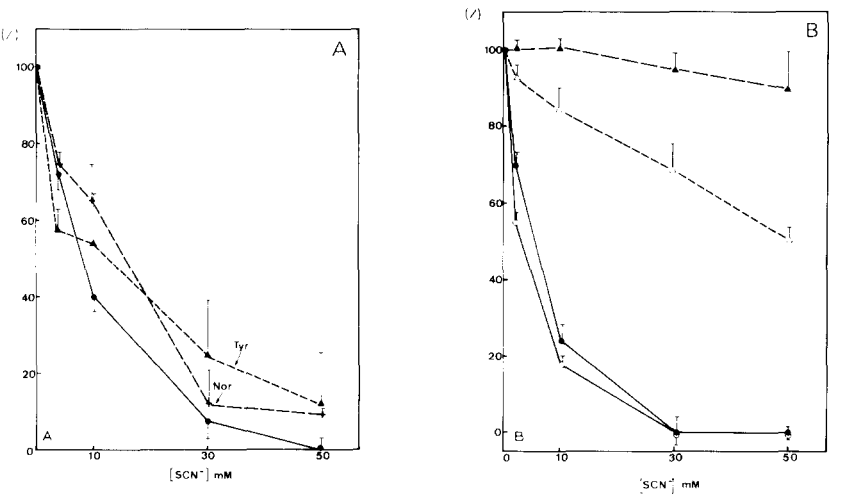


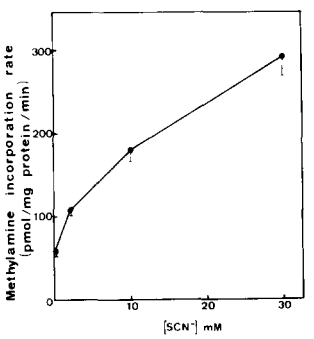
Fig. 1. Effect of KSCN on ATP-induced OX-V effect (solid line) and monoamine uptake (dotted line). A. Effect on granules. The incubation mixture contained ATP (5 mM) and $MgSO_4$ (2.5 mM) in 0.3 M sucrose, 20 mM NaOH/Hepes, pH 6.5, at 25°C. For fluorescence measurements (●), the granule and OX-V concentrations were 0.2 mg protein/ml and 2 μM, respectively. The fluorescence decrease due to ATP-induced polarization was reversed by successive additions of KSCN and the results, corrected for dilution, are the mean (±S.E.) of three experiments. Control OX-V effect was 40% fluorescence decrease. For uptake experiments, granules (1.1 mg protein/ml) were incubated at 25°C in a medium containing 0.3 M sucrose, 20 mM NaOH/Hepes, pH 6.5, 5 mM ATP, 2.5 mM $MgSO_4$ and various KSCN concentrations. After a 30 s incubation 100 μM labelled noradrenalin (+) or tyramine (▲) were added and aliquots were withdrawn during the linear period of uptake. Controls were 825 (+) and 165 (▲) pmol/mg protein per min. Uptake in absence of ATP was substracted. Points are mean (±S.E.) of three determinations. B. Effect on ghosts. The same experiment was performed on ghosts prepared in either 5 mM (●, ▲) or 50 mM (○, △) NaOH/Hepes, pH 7.0, the buffer of the incubation mixture being that used for ghost preparation. Ghost concentrations were 0.13 (●), 0.25 (○), 0.6 (▲) and 0,5 (△) mg protein/ml. Controls were 31 (●) and 35 (○) % for the fluorescence decrease and 1.65 (▲) and 2.6 (△) nmol/mg protein per min for noradrenalin uptake. Uptake in absence of ATP was substracted. Points are mean (±S.E.) of three determinations.

the concentration dependence of this effect was similar to that observed on the $\Delta\psi$. The ATP-dependent uptake of tyramine, which has characteristics in common with that of noradrenalin [30] was also blocked by SCN^-. On the other hand, with ghosts, uptake inhibition was less pronounced (Fig. 1B) and required ghosts prepared in strongly buffered solutions such as 50 mM Hepes. A better correlation of polarization and uptake inhibitions was observed at low (50 μM) ATP concentration (data not shown).

Since the collapse of the $\Delta\psi$ induced an inhibition of monoamine uptake which increased with the buffering capacity of the internal medium (granules > ghosts prepared in 50 mM Hepes > ghosts prepared in 5 mM Hepes), the effect of SCN^- on the internal pH of energized granules or ghosts was investigated. The pH gradient between the internal and external sides of the membrane was followed by measuring the uptake of methylamine. The rate of methylamine uptake by ghosts prepared in 50 mM Hepes was enhanced by SCN^- (Fig. 2), thus showing acidification of the ghost interior under these conditions. Such an acidification in presence of SCN^- was greater in ghosts prepared in 5 mM Hepes and was not seen in intact granules (data not shown).

Effect of cation efflux on the ATP-induced uptake of noradrenalin by ghosts

Cation efflux as well as anion influx should decrease the ATP-induced $\Delta\psi$ while not affecting, or even increasing, the ΔpH. Since the granule membrane is impermeant to most of the cations [20], ghosts were prepared in high K^+, incubated with valinomycin and added to low K^+ media. In such preparations K^+-efflux was expected to compensate for the proton influx generated by the pro-



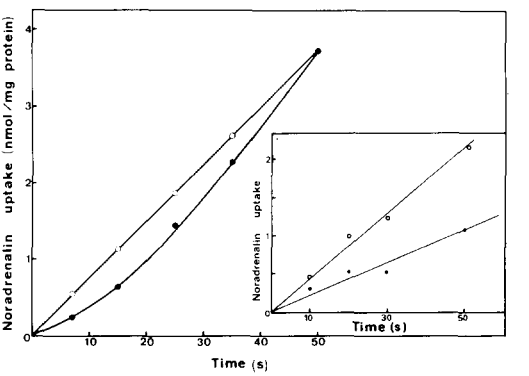


Fig. 2. Effect of KSCN on the ATP-induced methylamine uptake by ghosts. Ghosts (1.0 mg protein/ml) prepared in 50 mM NaOH/Hepes (pH 7.0) were incubated at 25°C with [^{14}C]methylamine (10 μM) and KSCN in a mixture containing ATP (5 mM), $MgSO_4$ (2.5 mM), 0.3 M sucrose and 20 mM NaOH/Hepes (pH 7.0). Uptake rates determined from the linear period of methylamine accumulation (at least 60 s) were plotted as a function of KSCN concentration. Control in absence of ATP was substracted. Points are mean (±S.E.) of three determinations.

Fig. 3. Effect of K^+-efflux on the ATP-induced noradrenalin uptake by ghosts. Ghosts (1.0 mg protein/ml) prepared in 5 mM NaOH/Hepes (pH 7.0) containing 10 mM K_2SO_4 were incubated at 37°C with (—)-[7,8-^{3}H]noradrenalin (100 μM) in presence (•) or in absence (○) of valinomycin (10 μM), in the mixture described in Fig. 2. Final external K_2SO_4 concentration was 0.33 mM. ATP and $MgSO_4$ were as in Fig. 1. A longer lag was observed when the experiment was repeated with 100 μM ATP and at 20°C (Inset).

ton pump and to induce a lag in the positive transmembrane polarization. The lag observed in the ATP-induced noradrenalin uptake (Fig. 3) was attributed to such a lag of polarization. No effect was observed over longer periods of time (from 1 to 15 min, data not shown), as expected from the transient nature of the K^+-efflux. At lower ATP concentration (100 μM) and temperature (20°C) a longer lag was observed (Fig. 3, inset), presumably because of the decrease of the H^+-pump turnover.

Effect of an imposed transmembrane potential on the ΔpH-driven noradrenalin uptake

If the $\Delta\psi$ participates in the force driving noradrenalin uptake, an artificially imposed $\Delta\psi$ such as that obtained by diffusion of K^+ through valinomycin-treated ghost membrane should stimulate uptake. In absence of any other electrogenic process the transmembrane potential may be assimilated to the K^+ diffusion potential given by the Nernst equation. Such a $\Delta\psi$ was unable to drive noradrenalin uptake on its own (see below); however, in the presence of a ΔpH the rate of the ΔpH-driven noradrenalin uptake was increased by a positive potential (Fig. 4A) and decreased by a negative one (Fig. 4B). Both the ΔpH and the $\Delta\psi$-dependent fractions of the uptake were blocked by tetrabenazine, a specific inhibitor of catecholamine uptake [21]. In contrast, the ΔpH-induced uptake of tyramine was not stimulated by a positive $\Delta\psi$ (Fig. 5). This lack of effect in the tyramine case might be attributed to technical reasons because of the rapidity of the uptake. It might more probably result from the fact that this process is not carrier-mediated [30].

Effect of the imposed potential magnitude

With ghosts prepared in 0.6 mM K_2SO_4 and in absence of valinomycin,

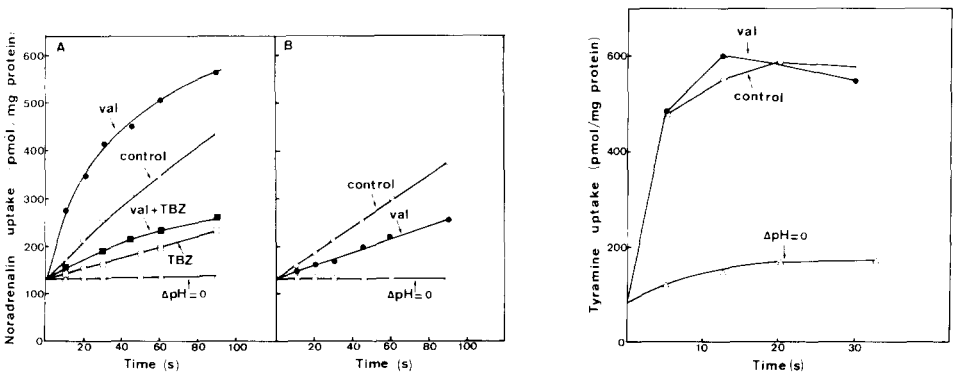


Fig. 4. Effect of a valinomycin-induced $\Delta\psi$ on the Δ pH-driven noradrenalin uptake. Ghosts (1.0 mg protein/ml) prepared in 5 mM NaOH/Hepes buffer (pH 7.0) and 10 mM K_2SO_4 were incubated at 37°C in media containing K_2SO_4 50 mM in A or 0.33 mM in B, 0.3 M sucrose, 20 mM NaOH/Hepes either at pH 8.0 (●, ○, ■, □) or at pH 7.0 (△), 10 μM [^{3}H]noradrenalin and, where indicated, 10 μM valinomycin (●, ■) and/or 10 μM tetrabenazine (□, ■). The same experiment has been repeated more than twenty times with similar qualitative results. Addition of 5 μM valinomycin to the pH 7.0 incubation mixture (ΔpH = 0) (△) did not change the results. val, valinomycin; TBZ, tetrabenazine.

Fig. 5. Effect of a valinomycin-induced $\Delta\psi$ on the ΔpH-driven tyramine uptake. Experimental conditions as in Fig. 5A [^{14}C]tyramine was 10 μM.

increasing the external K^+ concentration from 6 to 100 mM had no effect on the initial rate of the ΔpH-driven noradrenalin uptake (Fig. 6). K^+ concentrations higher than 100 mM increased the uptake rate, thus suggesting that high concentration gradients induced positive transmembrane potentials even in the absence of ionophore. In presence of valinomycin, external K^+ in the low concentration range (6—100 mM) effectively stimulated the initial rate of uptake, the effect increasing linearly with the logarithm of the ion concentration. The magnitude of the imposed $\Delta\psi$ was calculated by the Nernst equation, as described in Materials and Methods and it was concluded that the uptake enhancement was proportional to the $\Delta\psi$ in the 0—75 mV range (Fig. 6).

Variation with ΔpH of the valinomycin-induced enhancement of uptake

The above results (Fig. 6) were used to determine for each preparation the external K^+ concentration which was expected to give the optimal valinomycin-induced enhancement of uptake. The effect of the imposed $\Delta\psi$ was then analyzed as a function of the external pH (Fig. 7) and determination of the internal pH by the [^{14}C]methylamine uptake technique (as described in Materials and Methods) allowed estimation of the pH gradient. The valinomycin effect increased with the Δ pH up to 1.6, at which it levelled off. It has to be noted that a minimal ΔpH (0.3—0.6 pH unit in separate experiments) was required to observe the valinomycin effect, thus suggesting that a ΔpH was a prerequisite for monoamine uptake. To substantiate this point two types of experiment were undertaken. The magnitude of the $\Delta\psi$ was first increased by preparing ghosts at pH 7.0 in K^+-free media and suspending them at their internal pH (pH 6.5, measured as described in Methods) in buffers containing from 2.5 to

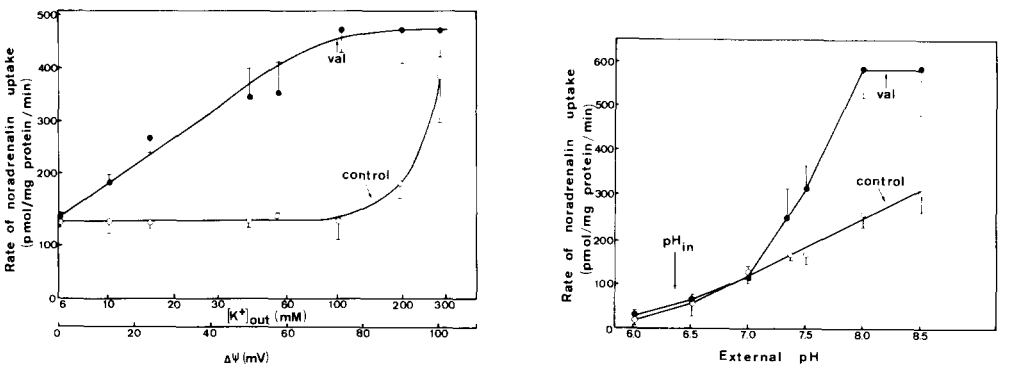


Fig. 6. Variation with $\Delta\psi$ of the ΔpH-driven noradrenalin uptake. Ghosts (2.4 mg protein/ml) prepared in 0.60 mM K_2SO_4 in 5 mM NaOH/Hepes (pH 7.0) were incubated at 37°C with 10 μM [^{3}H]noradrenalin, 0.3 M sucrose, 20 mM NaOH/Hepes (pH 8.0) and K_2SO_4 in presence (●) or in absence (○) of 10 μM valinomycin. Uptake was measured at 10, 20, 30 and 50 s and was linear for at least 30 s. Control values corresponding to ΔpH = 0 and $\Delta\psi$ = 0 were substracted. The internal K^+ concentration determined as described in Materials and Methods was 6 mM. Points are mean (±S.E.) of three determinations.

Fig. 7. ΔpH-dependence of the valinomycin-induced uptake enhancement. Ghosts were prepared in 0.3 mM K_2SO_4 in Tris-succinate buffer (pH 6.4). They were incubated at a concentration of 1.15 mg protein/ml with 10 μM [^{3}H]noradrenalin, 0.3 M sucrose, 20 mM K_2SO_4, 25 mM Tris-succinate at various pH levels in presence (●) or in absence (○) of valinomycin (10 μM), at 37°C. Uptake was linear for 40 s. The ghost internal K^+ concentration and internal pH, measured as described in Materials and Methods, were 3.1 mM and pH 6.4, respectively. Points are mean (±S.E.) of three determinations.

TABLE I

IMPORTANCE OF A ΔpH IN THE VALINOMYCIN-INDUCED UPTAKE

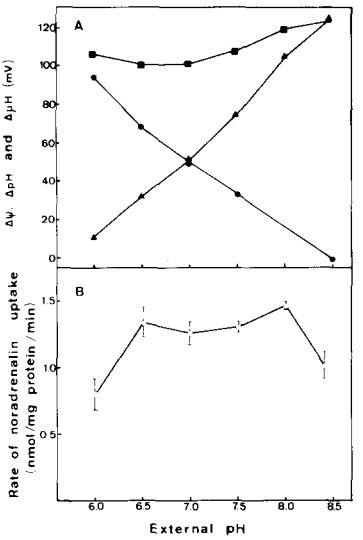
Ghosts were prepared in 0.6 mM K_2SO_4, 5 mM NaOH/Hepes (pH 7.0). Where indicated they were preincubated for 15 min at 37°C at a final pH of 7.5 in 25 mM NaOH/Hepes (Expts. 3 and 4) or in 2.5 mM NaOH/Hepes (Expts. 5 and 6). They were added to incubation media containing 10 μM noradrenalin, 0.3 M sucrose, 25 mM K_2SO_4, 25 mM NaOH/Hepes (final pH as indicated) and 50 mM $(NH_4)_2SO_4$ where indicated. For each experiment, noradrenalin uptake in presence or in absence of 5 μM valinomycin was measured at 20, 40, 60 and 90 s. and was corrected for time-independent uptake. Valinomycin-induced enhancement of uptake is mean (±S.E.) of values at 40, 60 and 90 s. Ghosts concentration was 1.0 mg of protein per ml.

Experiment number	Preincubation at pH 7.55	External pH during uptake	$(NH_4)_2SO_4$ 50 mM	Valinomycin-induced enhancement of uptake (%)
1	—	7.48	—	152 ± 30
2	—	7.48	+	10 ± 3.5
3	+	7.50	—	−4 ± 10
4	+	7.50	+	5 ± 11
5	+	8.02	—	133 ± 36
6	+	8.02	+	−16 ± 14

100 mM K^+. Such transmembrane potentials did not drive monoamine uptake when compared to controls performed either at high external K^+ in absence of valinomycin or at null external K^+ (data not shown). In the second type of experiment (Table I), ghosts prepared at pH 7.0, which showed a valinomycin-induced enhancement of uptake when assayed at pH 7.5 (Expt. 1), lost this property when first preincubated at pH 7.5 for 15 min at 37°C, to discharge the pH gradient (Expt. 3). The preincubation had no deleterious effect on the ghost preparation, since a valinomycin effect was again observed when the external pH was raised to pH 8.0 at the end of the preincubation period (Expt. 5). Ammonium sulfate (50 mM) which discharged the pH gradient without affecting the $\Delta\psi$ (as observed by ANS fluorescence measurements) also blocked the valinomycin-induced enhancement of uptake in these experiments (Expts. 2, 4 and 6).

$\Delta\mu_{H^+}$-dependence of the ATP-induced noradrenalin uptake

Since both the $\Delta\psi$ and the ΔpH appeared to be involved in the ATP-induced catecholamine uptake, the role of the $\Delta\mu_{H^+}$ was then investigated. The ΔpH, $\Delta\psi$ and $\Delta\mu_{H^+}$ values of energized chromaffin granules were determined at various pH (Fig. 8A) and were compared to the ATP-induced noradrenalin uptake at the same pH (Fig. 8B). This experiment was performed on granules, since their high buffering capacity limits the variation of their internal pH (the variation, in the presence of ATP, being from 5.8 (at pH 6.0) to 6.3 (at pH 8.5)). In the same pH range the $\Delta\psi$ of energized granules decreased linearly with the pH (Fig. 8A), as previously reported [8,31]. The $\Delta\mu_{H^+}$ of the energized granules, derived from $\Delta\psi$ and ΔpH values, did not vary with the external pH (Fig. 8A). Since in that pH range the ATPase activity was independent of the external pH (Ref. 7, and data not shown), this result confirmed that the $\Delta\mu_{H^+}$ (about 110 mV) described the proton-motive activity of the H^+-translocase, as proposed by Mitchell [22]. The initial rate of noradrenalin uptake varied only



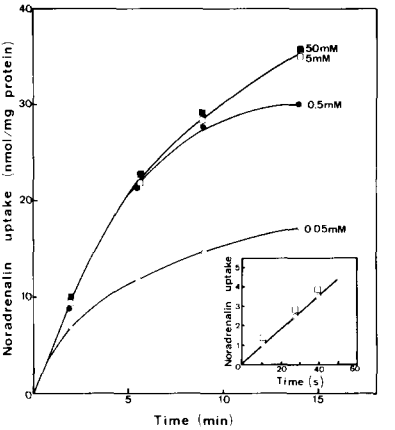


Fig. 8. Effect of the external pH on ΔpH, $\Delta\psi$ and noradrenalin uptake by energized granules. A. $Z\Delta$pH (▲) expressed in mV and $\Delta\psi$ (●) were measured as described in the experimental section and $\Delta\mu_{H^+}$ (■) was derived from ΔpH and $\Delta\psi$. Experiments were performed in duplicate, S.E. being always less than 2 mV. B. Noradrenalin uptake (○) was measured after a 1 min preincubation of granules (0.8 mg protein/ml) in 5 mM ATP, 2.5 mM $MgSO_4$, 0.3 M sucrose and 25 mM Tris-succinate at the indicated pH. [^{3}H]-Noradrenalin (100 μM) was then added and aliquots withdrawn after 0.5, 1 and 2 min. Controls performed in presence of 10 μM tetrabenazine were substracted. Points are mean (±S.E.) of three determinations.

Fig. 9. ATP-induced noradrenalin uptake by ghosts with different internal buffering capacity. Ghosts were prepared from the same granule preparation in 0.05 (○), 0.5 (●), 5 (□) and 50 (■) mM KOH/Hepes (pH 7.0). They were incubated for various times at about 1 mg protein/ml in 100 μM [^{3}H]noradrenalin, 5 mM ATP, 2.5 mM $MgSO_4$, 0.3 M sucrose and 5 mM KOH/Hepes (pH 7.0), at 37°C. The initial part of the uptake (inset) is presented only for ghosts prepared in 0.05 (○) and 5 (□) mM Hepes but the other preparations gave similar results. Controls in presence of 10 μM reserpine were subtracted. The preparations gave ATP-induced OX-V effect of 16, 22, 22 and 17% for preparations in 0.05, 0.5, 5 and 50 mM Hepes, respectively, indicating that the change in noradrenalin uptake was not associated with a change in the H^+-pump activity.

slightly with the pH (Fig. 8B) and therefore appeared to be clearly correlated with the $\Delta\mu_{H^+}$.

Role of anions in the catecholamine uptake

The anions of the sucrose medium used for optimal catecholamine uptake were not directly involved in the mechanism of that process, since they could be manipulated without affecting the initial rate of uptake: blocking ATP uptake by atractyloside, suppressing SO_4^{2-} by the use of the Mg^{2+} salt of ATP and associating noradrenalin with permeant (chloride, tartrate, acetate), slightly permeant (sulfate) or impermeant (phosphate) anions did not inhibit significantly the rate of noradrenalin uptake, which was 87% of the control in the standard medium. Since transport of anions does not seem to be directly involved in the catecholamine uptake, the counter-ion of the internal catecholamine cation should be provided by the vesicle matrix. With ghosts prepared in media containing anionic buffer at different concentrations, noradrenalin was taken up at the same initial rate (Fig. 9, inset) but the time for reaching plateau

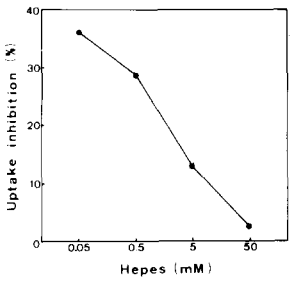


Fig. 10. Inhibition of noradrenalin uptake by atractyloside. Ghosts prepared in various concentrations of Hepes buffer as described in the legend of Fig. 9 were incubated for 11 min with 200 μM atractyloside in the medium described in the same legend. The drug had only a limited effect on the initial rate of uptake. Points are mean of two determinations.

was shorter and the plateau value was lower in the case of the less-buffered ghosts (Fig. 9). The limited uptake observed with ghosts prepared in 0.05 mM Hepes was attributed to an alkalinization of the vesicle interior associated with noradrenalin entry, resulting in an inhibition of the active transport process. According to this hypothesis, an uptake of anions which would delay the alkalinization would maintain catecholamine uptake. That the ATP anion could play that role was shown by an experiment performed on ghosts with various buffering capacity. The plateau fraction of the noradrenalin transported which was inhibited by atractyloside increased when the buffering capacity decreased (Fig. 10), suggesting that alkalinization was partially reversed by a proton-ATP cotransport.

Discussion

$\Delta\psi$-dependence of noradrenalin uptake

The participation of transmembrane potentials in monoamine uptake has been and still is a matter of controversy [11,12,15,23]. The present communication adds some new evidence to the $\Delta\psi$-dependence of noradrenalin uptake. Selective inhibition of the positive $\Delta\psi$ generated by the H^+-translocase, either by anion influx (Fig. 1) or by cation efflux (Fig. 3) resulted in an inhibition or a lag of ATP-induced monoamine uptake. It has to be pointed out that the former experiment gave more conclusive results when performed on granules than on ghosts. This difference is attributed to pH changes (Fig. 2) associated with thiocyanate uptake, since larger inhibitions were observed when ghosts were prepared in concentrated (50 mM) buffer (see below). We have also found that the ATP-dependence of ATP hydrolysis (in presence of oligomycin), membrane polarization (monitored by the OX-V effect) and initial rate of monoamine uptake were similar (characterized by a K_m (ATP) of, respectively, 70, 72 and 86 μM (data not shown)), as required if monoamine uptake is causally linked to the transmembrane polarization induced by the ATP-dependent proton pump.

The effect of diffusion potentials superimposed on the ΔpH-driven uptake strongly supports the involvement of transmembrane potentials in the noradrenalin uptake process. Inside positive potentials (which had the polarity of

the $\Delta\psi$ generated by the H^+-translocase) increased the rate of uptake, whereas negative $\Delta\psi$ decreased it. To observe this effect, which has been repeatedly seen in more than 20 experiments, the K^+ concentration gradient had to be adjusted to optimize the valinomycin effect, since high K^+ concentration gradients generated a $\Delta\psi$, even in absence of valinomycin (Fig. 6). If the $\Delta\psi$ is participating in the driving force of catecholamine uptake, it should increase not only the rate of uptake but also its extent, to which it is thermodynamically related. Under the conditions used in Fig. 4 the plateau values obtained in the presence of a $\Delta\psi$ were not always higher than the control. This observation can be explained in that the major part of the $\Delta\psi$ had a lifetime shorter (40 s) than that of the pH gradient (4 min) (Scherman and Henry, unpublished experiment), thus resulting in a plateau value at longer times related mainly to the ΔpH.

ΔpH and $\Delta\mu_{H^+}$-dependence of monoamine uptake

It is well established that an imposed ΔpH can drive monoamine uptake [11—13]. In presence of permeant anions the H^+-pump can generate a ΔpH [4,5] and the limited inhibition by SCN^- of noradrenalin uptake by ghosts prepared in 5 mM Hepes is attributed to such a ΔpH-driven uptake, the collapse of the $\Delta\psi$ being compensated for by an increase of the pH gradient. In our experiments, the exact value of the ΔpH was not known, since with the technique used (uptake of [^{14}C]methylamine followed by filtration) results were essentially qualitative due to possible loss of methylamine during filtration and to the difficulty of estimating the internal volume of the filtered material. The limited ΔpH generated by the H^+-pump in absence of SCN^- might play a role in noradrenalin uptake (see below, mechanism of uptake), but the rate of uptake did not depend dramatically on the anions present in the sucrose incubation medium.

Both ΔpH and $\Delta\psi$ affect monoamine uptake and the analysis of the pH-dependence of the ATP-induced uptake showed that the initial rate of monoamine transport by chromaffin granules was correlated with the $\Delta\mu_{H^+}$ which is quite independent of the external pH (Fig. 8). Surprisingly, data on the pH profile of monoamine uptake are scarce [24,25] and have been obtained under different conditions. Taugner [24] used ghosts prepared in unbuffered media and in the communication of Aberer et al. [25] rates of uptake were not considered.

The proton motive force of the H^+-translocase derived from Fig. 8 (100—120 mV) is somewhat lower than the figures of 138 and 150 mV previously reported for granules [10] and ghosts [5] respectively. These values have to be compared to the $\Delta\mu_{H^+}$ inducing maximal rates of uptake, as derived from Figs. 6 and 7, which describe the effects of imposed ΔpH and $\Delta\psi$. Similar values were obtained when ΔpH varied at fixed $\Delta\psi$ (155 mV) and when $\Delta\psi$ varied at fixed ΔpH (140 mV), indicating that beyond this value the rate of uptake was limited by other factors. The fact that the proton-motive force of the H^+-translocase is not far from this value suggests nearly optimal energetic conditions for the ATP-dependent monoamine uptake.

Mechanism of the monoamine uptake

The $\Delta\mu_{H^+}$-dependent step is likely to involve the monoamine carrier, since

the processes which were sensitive to the $\Delta\psi$ were those shown to be carrier-mediated, i.e., the ATP-driven uptake of noradrenalin and tyramine and the ΔpH-driven uptake of noradrenalin [30]. The ΔpH-driven uptake of tyramine, which had been shown to involve nonspecific diffusion of the amine through the membrane, was not activated by a $\Delta\psi$.

A ΔpH-sensitive step seems to be involved in addition to the $\Delta\mu_{H^+}$-dependent one, since a $\Delta\psi$ alone did not drive monoamine uptake: in the case of Fig. 7 the imposed $\Delta\psi$ of 60 mV may have been too weak to drive the electrogenic process in the absence of ΔpH; however, with ghosts prepared in K^+-free media, the imposed $\Delta\psi$, which was unable to drive an uptake at null ΔpH, was expected to be higher than a 80 mV value corresponding to a $\Delta\mu_{H^+}$ inducing significant uptake in presence of a ΔpH (Fig. 6). This result was confirmed by the experiment described in Table I. The ΔpH requirement differs from recent results by Njus and Radda [17] observed under different conditions and with a different preparation. The origin of this discrepancy is being actively investigated. In another recent publication [26] Johnson and Scarpa have described an ATP-dependent monoamine uptake by ghosts under conditions in which the ΔpH was very limited. Nevertheless, it has to be noted that in their experiments decreasing the ΔpH decreased dramatically the rate of uptake, even though the $\Delta\mu_{H^+}$ was constant.

Njus and Radda [16] and Johnson and Scarpa [10] have presented mechanisms whereby uptake of one amine either in neutral or in cationic form resulted in net efflux of one positive charge, thus accounting for the role of the potential. In the model proposed by Johnson and Scarpa, the positive $\Delta\psi$ drove the inward translocation of a complex involving a neutral catecholamine and a negatively charged carrier. Dissociation of the complex was favoured on the acidic side of the membrane on which the transported amine was protonated. This first step was thus $\Delta\mu_{H^+}$-dependent, whereas the second one, transport of the neutral protonated carrier from the inner to the outer side of the membrane, was ΔpH-dependent. Such a model accounts for our data since it involves both a $\Delta\mu_{H^+}$ and a ΔpH-sensitive step and our results (Fig. 8) suggest that translocation of the catecholamine-carrier complex might be the rate-limiting step. Another equivalent model involves a ΔpH-driven translocation of a neutral amine-neutral carrier complex, the $\Delta\mu_{H^+}$-driven step being the outward translocation of the positively charged protonated carrier. Transport of the cationic form of the catecholamine can be described by a formally similar model. Nevertheless, the consequences of such a model are different. The translocation of the neutral complex of a protonated amine with a negatively charged carrier would be neither $\Delta\psi$- nor ΔpH-dependent, whereas the force driving the second step, outward transport of two protons, would be proportional to $\Delta\psi + 2Z\Delta$pH. We consider such a mechanism to be unlikely for the following reasons: (i) Johnson and Scarpa [10] have provided evidences in favour of the transport of the neutral form of catecholamines; (ii) our data (Fig. 8) indicate a driving force proportional to $\Delta\mu_{H^+}$ rather than $\Delta\psi + 2Z\Delta$pH; (iii) this model does not account for the ΔpH-requirement of $\Delta\psi$-driven uptake.

Charge and H^+ balance sheet

According to electrodissipative models [10,16], the electroneutrality will be maintained during catecholamine uptake if the efflux of charges through the carrier be strictly compensated for by the proton influx through the H^+-pump. In the model discussed, the carrier exchanges one proton for a neutral amine which is protonated inside of the vesicle, resulting in the loss of two protons per amine accumulated, whilst only one proton is pumped by the H^+-translocase. Thus electroneutral uptake results in alkalinization of the vesicle interior. The experiment described in Fig. 10 suggests such an alkalinization. In the physiological situation two factors might limit this phenomenon. Anion uptake, though not directly involved in the mechanism of amine uptake, would allow non electrogenic pumping of protons [5]. In this regard, ATP-induced ATP uptake [25,27,28] would be important (see Fig. 11) since transport of one nucleotide would allow accumulation of three to four monoamines (close to the nucleotide to amine ratio found in the mature granule [29]) without any change of the internal pH. A second factor is the high buffering capacity of the granule matrix, due to large amounts of non-diffusible anions [13]. These molecules are inserted in the granule during a maturation step such that counterion accumulation should precede catecholamine uptake [14].

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