NET UPTAKE OF CATECHOLAMINES INTO ISOLATED CHROMAFFIN GRANULES DEMONSTRATED BY A NOVEL POLAROGRAPHIC TECHNIQUE

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1. Introduction

The recognition of the importance of chromaffin granules both in the control of adrenal catecholamine homeostasis and as an excellent model for the broader investigation of biogenic amine transport has led to the development of procedures for the routine isolation of chromaffin granules in high yield and purity. Biochemical analysis of the composition of granules isolated in vitro indicates that they contain large quantities of catecholamines (>1 μmol/mg protein) [1,2]. The mechanism by which the granule accumulates and stores catecholamines against an apparent concentration gradient has recently become an increasingly active area of biologic investigation, and a molecular model for the active accumulation of catecholamines has been proposed. The model is based upon the documented existence of a H^+-translocating ATPase within the chromaffin granule membrane, the generation of a transmembrane pH gradient (ΔpH, inside acidic), and transmembrane potential (ΔΨ, inside positive), and the coupling of the resulting $\Delta\bar{\mu}_{H^+}$ to a catecholamine carrier [3–13].

The transport studies have for the most part been undertaken by measurement of the steady-state distribution of radiochemically-labeled biogenic amines across the chromaffin granule membrane. The early investigations were performed with intact chromaffin granules which were pelleted or filtered from the incubation medium [5–14]. Due to the undesirably high amine content and endogenous proton gradient of intact granules, however, subsequent definitive studies were performed using chromaffin ghosts purified by several procedures [7,8,12–14]. While use of the ghost preparations has contributed significantly to the elucidation of the mechanism of catecholamine transport, the intrinsic limitations of the radiochemical amine distribution methodology have greatly limited further characterization of the precise molecular mechanism of coupling of catecholamine transport to the $\Delta\bar{\mu}_{H^+}$. In addition, despite the rather sophisticated analysis of kinetic catecholamine fluxes precent in the literature, the objection has been raised that a basic criterion for demonstration and study of catecholamine transport, that of *net* accumulation into granules or ghosts, has yet to be demonstrated [16,17].

This study presents two techniques which overcome many of the limitations of the methodologies available for measurement of catecholamine accumulation.

(1) Accumulation of individual species of catecholamines was quantitated using high-pressure liquid chromatography after filtration of the ghosts from the incubation medium;
(2) The online kinetic influx and efflux of catecholamines across the chromaffin ghosts membrane was monitored using a potentiometric electrode.

The results indicate that *net* epinephrine accumulation into isolated chromaffin ghosts does in fact occur, and provides further evidence that the $\Delta\bar{\mu}_{H^+}$ may play a central role in the mechanism of catecholamine accumulation in vivo.

2. Experimental

Chromaffin ghosts were prepared from bovine adrenal medulla as in [13,18]. In brief, freshly isolated chromaffin granules purified from other subcellular organelles by centrifugation through an isotonic density gradient of Percoll [18] were lysed by suspen-

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sion in a large volume of hypotonic media, the membranes were pelleted, and the lysis procedure repeated. Ghosts were formed by resuspension of the membranes in 4 ml buffered 185 mM KCl, followed by overnight dialysis against 4 l of the same medium. After one washing in fresh medium the ghosts were used immediately in experiments and assays. Protein was measured as in [19], using bovine serum albumin as the standard.

The equilibrium distribution of [^{14}C]methylamine (8.8 μM) and $^{3}H_2O$ across the chromaffin granule membrane was utilized to measure the ΔpH across the chromaffin ghost membrane. This method has been found to be highly reproducible over a wide range of experimental conditions, with minimal binding observed [9,13].

The experimental procedure for measurement of on-line kinetic accumulation of catecholamines into isolated chromaffin ghosts, and the advantages and limitations of this method, are detailed in [20], based on a modification the methods in [21–23]. In brief, amines were measured as their reversible oxidation products detected by a glassy carbon electrode (Bioanalytical Systems, W. Lafayette IN) set at a potential of 0.50 V *vs* an Ag/AgCl reference electrode (Bioanalytical Systems, W. Lafayette IN) with an auxiliary steel electrode. The glassy carbon electrode was maintained at that fixed potential with a model CV-1A amperometric controller (Bioanalytic Systems, W. Lafayette IN) connected to a standard single pen recorder. The assembly was housed in a Faraday cage to optimize the signal-to-noise ratio. The experimental medium consisted of 185 mM KCl, 30 mM Hepes buffer (pH 6.80), 2.5 mM MgATP, and chromaffin ghosts (0.3–0.4 mg protein/ml) incubated at 37°C with constant stirring. Calibration was accomplished with successive additions of 5 μM epinephrine dissolved in 30 mM Hepes (pH 6.80) and was found to be linear over 2–40 μM. Endogenous catecholamine content of the reaction mixture was always <2 μM. Intensive investigation of the electrode properties in the system has indicated that $>98\%$ of the electrode response is due to catecholamine fluxes (not shown).

Alternatively, epinephrine concentrations in the supernatants were measured by high pressure liquid chromatography using a model 600 solvent delivery system (Water Assoc., Milford MA) with a model U6K sample injector (Waters Assoc.) and a model 441 absorbance detector. The chromatographic separation of epinephrine from all the other components in the reaction mixture was achieved at 20°C on a Bondapak C18 reverse-phase column (10 μM particle size range, 300 X 2.9 mm i.d., from Waters Assoc.) supplemented with a small precolumn (30 X 2.9 mm i.d.) filled with Bondapak C18 reverse phase material. The mobile phase, consisting of 500 mM ammonium phosphate (pH 4.4), was pumped at a flow rate of 2 ml/min (1800 lb/in.^{2}). The retention time of epinephrine was 2.4 minutes and its absorbance was measured at 214 nm (0.005 AUFS) and displayed in a strip chart recorder.

[^{14}C]Methylamine (48.1 mCi/mmol), $^{3}H_2O$ (1 mCi/g), and [^{14}C]polydextran (1.11 mCi/g) were purchased from New England Nuclear (Boston MA). Epinephrine bitartrate, tyramine–HCl, and the standard reagents were purchased from Sigma Chemical Company (St Louis MO).

3. Results

The large magnitude net accumulation of epinephrine into isolated chromaffin ghosts is demonstrated by two complementary techniques in fig.1. With the

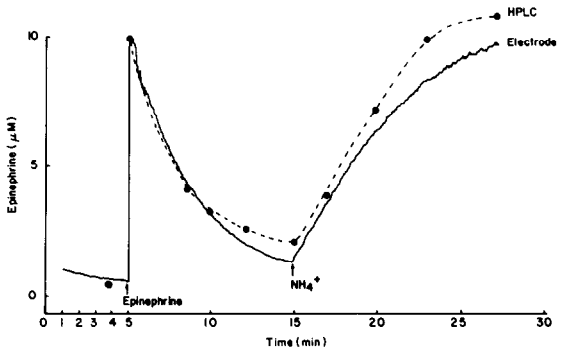

Fig.1. The kinetic measurement of epinephrine accumulation into chromaffin ghosts by potentiometric techniques and HPLC. The reaction mixture contained 185 mM KCl, 30 mM Hepes (pH 6.80) and chromaffin ghosts (0.34 mg protein/ml). The total volume was 2.1 ml. The catecholamine, reference, and auxiliary electrodes were allowed to equilibrate in the reaction chamber; 2.5 mM MgATP was added to initiate the reaction and 10 μM epinephrine bitartrate and 20 mM $(NH_4)_2SO_4$ were added at the times indicated. For HPLC determination, 200 μl aliquots of the experimental medium were removed and centrifuged for 7 min in a model 4200 Eppendorf microcentrifuge at the times indicated, and the supernatant was immediately assayed for epinephrine (section 2). Temperature was maintained at 37°C using a water bath.

first technique (HPLC) aliquots of the incubation medium containing the ghosts were rapidly filtered through cellulose filters (0.45 μM, Millipore). The effusate was assayed for catecholamines by HPLC as described in section 2. In the second (electrode) a glassy carbon potentiometric electrode was utilized to measure the total free catecholamine concentration within the stirred suspension. The experimental conditions were identical for the two techniques. The catecholamine concentration in the medium after addition of ghosts as measured by both techniques was 1.8 ± 0.4 μM. Total endogenous catecholamines in the ghost preparation determined through HPLC was 7.1 (±1.3) nmol/mg protein and the measured amine was predominantly epinephrine, indicating that the large majority of the endogenous catecholamine content of the ghost preparation was confined to the extravesicular space.

The addition of ATP to ghosts suspended in KCl media resulted in the generation of a ΔpH, inside acidic, across the chromaffin ghost membrane due to the inwardly directed H^+-translocating ATPase and the relatively high membrane permeability of Cl– [5,13]. [^{14}C]Methylamine distribution used for measurement of the magnitude of the ΔpH across the chromaffin ghost membrane indicated that in this experiment the ΔpH reached a steady state value of 1.1 after 5 min. Five minutes after the addition of ATP, 10 μM exogenous epinephrine was added to the reaction mixture (fig.1,→). Disappearance of the amines from the media, corresponding to catecholamine uptake into the intravesicular space of the ghosts, is observed (subsequent to the arrow in fig.1). Of note is the fact that both techniques recorded the disappearance of the amine from the medium and, despite the different methodologies, produced nearly identical quantitative values. Ten minutes after the addition of epinephrine, accumulation reached steady-state conditions. If the accumulated amines were truly in equilibrium with the electrochemical proton gradient, collapse of the ΔpH should result in the consequent redistribution of the intravesicular amines. Complete collapse of the ΔpH can be achieved by addition of a large concentration of ammonia [9,13]. The addition of 40 mM ammonia to the incubation medium of the experiment shown in fig.1 resulted in a decrease of the ΔpH from 1.2–0.1 pH units (not shown), and (second arrow) a corresponding efflux of the amines to near baseline levels.

These experiments indicate that methodologies exist for the simultaneous measurement of both exogenous and endogenous amine fluxes into chromaffin ghosts and that large magnitude *net* accumulation of amines in response to the $\Delta\bar{\mu}_{H^+}$ does in fact occur. In addition, the experiments suggest that measurement of catecholamines by the polarographic electrode in a well-defined chromaffin ghost system may be of particular advantage due to ease of measurement, sensitivity, accuracy and ability to record kinetics, when compared to existing techniques. Dozens of polarographic experiments with a variety of ghost preparations indicate that the rate of epinephrine uptake by the ghosts is 10.5 (±3) nmol . min^{-1} . mg $protein^{-1}$ and that a steady state level of epinephrine accumulation >60 nmol/mg protein could be obtained.

To further investigate the possible utility of the polarographic electrode in the measurement of catecholamine fluxes, the polarographic technique for catecholamine uptake was applied to the kinetic measurement of catecholamine influx (fig.2) under

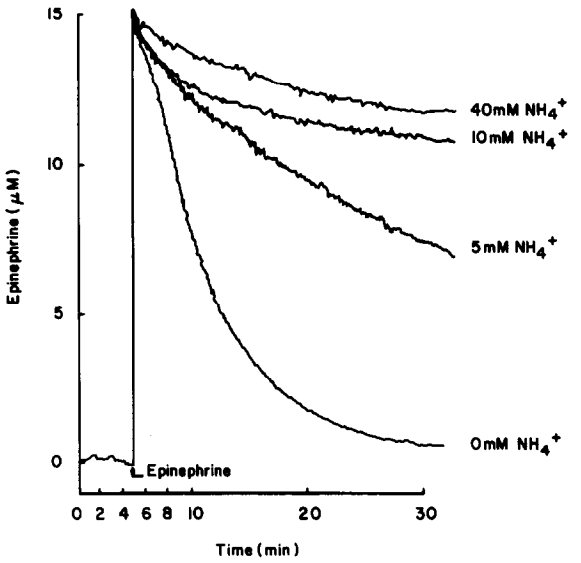

Fig.2. pH-dependent accumulation of epinephrine into chromaffin ghosts. To a mixture of 185 mM KCl, 30 mM Hepes (pH 6.80), chromaffin ghosts (0.32 mg protein/ml), and varying concentrations of $(NH_4)_2SO_4$ (shown superimposed), was added 2.5 mM MgATP to initiate generation of a ΔpH. Epinephrine bitartrate (15 μM) was added after 5 min had elapsed. The ΔpH in each experiment was measured by [^{14}C]-methylamine distribution 5 min after MgATP addition and was found to be 1.18 (0 mM NH_4^+); 0.70 (5 mM NH_4^+); 0.51 (10 mM NH_4^+); 0.20 (40 mM NH_4^+). Temperature was 37°C.

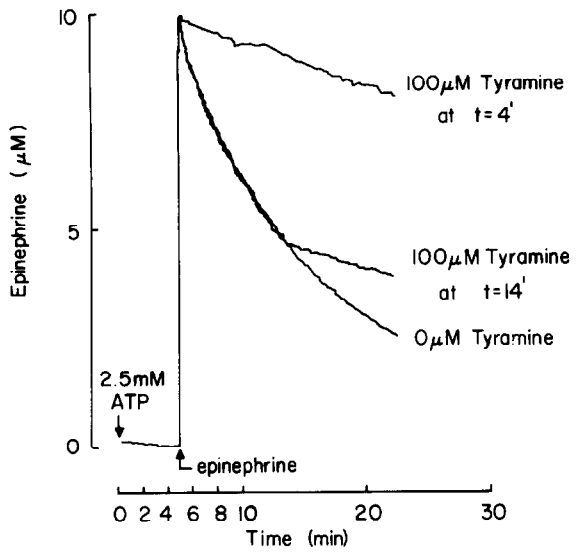

Fig.3. Competitive inhibition of epinephrine accumulation by tyramine in chromaffin vesicles. Chromaffin ghosts (0.38 mg protein/ml) were added to a reaction medium of 185 mM KCl and 30 mM Hepes. MgATP (2.5 mM), epinephrine bitartrate (10 μM), and tyramine–HCl (at the indicated concentrations) were added at the times indicated. Results of 3 expt are shown superimposed. Temperature was 37°C.

conditions wherein the ΔpH was varied from 1.8–0.2 pH units (not shown) by the addition of increasing concentrations of ammonia (0–40 mM). The data indicate that the rate and extent of catecholamine accumulation corresponds to the magnitude of the ΔpH and that even with very small ΔpH values significant net accumulation of the biogenic amine can be measured.

Central to the model for catecholamine accumulation into the chromaffin ghost is movement of catecholamines through the membrane mediated by a putative carrier molecule. This carrier is known to maintain selectivity toward structurally related biogenic amines [24,25]. One of the most potent inhibitors of carrier-mediated catecholamine uptake, tyramine, was thus investigated for its effect upon the rate and extent of epinephrine uptake using the polarographic technique (fig.3). As a monohydroxy phenylamine, tyramine present in the incubation medium (up to 5 mM) was not measured by the electrode (not shown). The addition of tyramine (100 μM) significantly reduced the rate and net accumulation of epinephrine when added before or after the addition of epinephrine. This observation is consistent with the notion that carrier-mediated transport is being measured with the electrode technique.

4. Discussion

It is now well-established that the electrochemical proton gradient is the driving force for amine uptake in chromaffin granules and ghosts, and recent investigations have dealt with attempts to further elucidate the molecular mechanism of the transport process through determination of the physico-chemical properties of the carrier and the species of catecholamine transported [5–13]. However, these sophisticated analyses have failed to unequivocally define a unique model for the transport process and, in fact, extremely opposite conclusions have been reached by investigators studying the same physico-chemical properties [26,27]. These controversies may arise from two major experimental limitations:

(1) Despite the in-depth analysis of data obtained for measurement of biogenic amine accumulation, one of the most fundamental criteria for the establishment of an active transport process, i.e., the existence of net uptake, has not yet been demonstrated. Thus, in all of these measurements, the exchange of intravesicular endogenous amines for exogenous amines, the possible metabolism or oxidation of the added amines, and the redistribution of gradients during preparative centrifugation are still unknown quantities.

(2) Nearly every major study of biogenic amine uptake into isolated chromaffin granules of ghosts has measured amine fluxes by utilizing distribution of radiochemically-labeled catecholamines. This method, however, is limited by its inability to detect:
(i) Movement of nonlabeled endogenous amines;
(ii) Any oxidation of metabolism of the exogenous amine (since the label is usually a carbon or hydrogen atom on the phenyl group).
Furthermore, due to the necessity for lengthy centrifugation to separate the granules or ghosts from the incubation medium, redistribution and/or aspecific binding of the amines during separation has become an eminent concern.

This study has attempted to answer these concerns in an effort to more accurately define the properties of biogenic amine transport. Two complementary techniques were developed which overcome many

of the limitations of previously described methods. Analysis of the biogenic amine by HPLC after filtration of the ghosts from the incubation medium permitted accurate, sensitive measurement of the endogenous and exogenous catecholamine content as the individual species, as well as any metabolite or oxidation products. Measurement of the free catecholamine concentration in the incubation medium by the polarographic electrode permitted for the first time an accurate on-line kinetic recording of catecholamine fluxes.

Application of these techniques to the study of epinephrine accumulation and release in the chromaffin ghost revealed that:

(i) Significant net accumulation of epinephrine into the chromaffin ghost occurs (fig.1–3);
(ii) Distribution of the accumulated biogenic amine was proportional to the ΔpH, with both the rate and extent of influx and efflux determined by the magnitude of the proton gradient (fig.1,2);
(iii) Consistent with a carrier-mediated process, epinephrine uptake was inhibited by a structurally-related amine, tyramine.

An ATP-dependent membrane carrier catalyzes a 1:1 exchange of an endogenous intragranular matrix amine for an exogenous amine [16]; it was concluded that ATP catalyzes exchange of catecholamines rather than net accumulation in intact granules [16]. Unfortunately, even previous transport studies utilizing chromaffin ghosts could not refute this conclusion as the only mechanism operative, due to the presence of residual endogenous amines and the measurement techniques utilized.

However, using the techniques described, these experiments unequivocally indicate that a mechanism does exist for the large magnitude net influx of catecholamines across the chromaffin ghost membrane against a concentration gradient. In addition, the measurement of on-line catecholamine fluxes through the electrode makes possible kinetic measurements of free amine concentrations and should prove a powerful new tool for the study of catecholamine transport.

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