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LARGE-SCALE PREPARATION OF PLASMA MEMBRANE VESICLES FROM PC-12 PHEOCHROMOCYTOMA CELLS AND THEIR USE IN NORADRENALINE TRANSPORT STUDIES

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Plasma membranes were isolated from rat pheochromocytoma cells (PC-12) grown in spinner culture. The rapid and simple isolation procedure consisted of a differential and isopycnic centrifugation (in a linear sucrose gradient) with the aid of a high capacity fixed angle rotor equipped with siliconized centrifuge tubes. The isolated membranes were closed and osmotically active vesicles (about 0.3 μm in diameter) with a mean intravesicular water space of 1.84 μl/mg protein. In the presence of an inward gradient of sodium chloride and an outward gradient of potassium, [^{3}H]noradrenaline (50 nM) was taken up and accumulated 550-fold (at 31°C). The uptake and accumulation of [^{3}H]noradrenaline was temperature-sensitive and inhibited by the tricyclic antidepressant desipramine. Membrane vesicles isolated from PC-12 cells represent a useful model for the investigation of the molecular mechanism of the neuronal noradrenaline transport system.

Introduction

PC-12 cells (clonal rat pheochromocytoma cells) exhibit properties of adrenergic neurones (for review see Greene and Tischler [1]). They possess a transport system for noradrenaline [2], which corresponds to the high-affinity amine transport system of adrenergic nerve terminals ('uptake$_1$' of Iversen [3]).

Some molecular properties of 'uptake$_1$' are not yet established because of the lack of a system in which a defined modification of the intraneuronal solute composition can be achieved. For example, the role of the intraneuronal K^+ in the transport of noradrenaline is unknown. However, extracellular K^+ is known to be inhibitory to the uptake of noradrenaline. Plasma membrane vesicles of adrenergic neurones would represent a simple system to study the effect of potential differences and electrochemical gradients across the plasma membrane on amine transport. Due to the heterogeneity of synaptosomes [4] and their limited viability [5], 'ghosts' obtained from synaptosomes are disadvantageous for this purpose. Hence, a method for the preparation of plasma membrane vesicles from a homogeneous population of neuronal cells (PC-12 cells) was developed. In this study a rapid and simple large-scale procedure for the isolation of osmotically sensitive plasma membrane vesicles is described; in addition, a micromethod for studies on the transport of noradrenaline into these plasma membrane vesicles is presented. A preliminary account of this work has appeared [6].

Materials and Methods

Cell culture

PC-12 cells, originally established by Greene and Tischler [7], were a gift of Dr. Hans Thoenen, Munich. They were initially grown on plastic cul-

Abbreviation: PMSF, phenylmethanesulfonyl fluoride.

ture flasks at 37 °C in a humidified atmosphere with 7% CO_2. The growth medium, which did not contain any antibiotics, consisted of 85% Dulbecco's modified Eagle medium (Gibco), supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum and 3.7 g/l $NaHCO_3$; the medium was changed every 2–3 days. The doubling time of the cells was about 4 days under these conditions. For subcultivation, nearly confluent cells were detached from the culture flask by a short exposure to trypsin (0.25% in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline), washed in culture medium and replated at a density of about 10^5 cells/cm^2.

For preparation of plasma membrane vesicles, cells were grown in spinner culture. To initiate spinner culture, cells were fed for 2 weeks with 85% RPMI (Gibco), supplemented with 5% fetal calf serum, 10% heat-inactivated horse serum and 2 g/l $NaHCO_3$ (no antibiotics were added). After detachment and washing (see above), cells were adjusted to a Bellco spinner culture flask (with two side arms). Initially, the pH of the medium was lowered to 7.6 by introducing CO_2. The stirrer was adjusted to 60 rpm. When the production of CO_2 by the cells caused a decrease of the pH of the medium below a value of 7.3, the caps of the side arms were slightly opened to allow the escape of CO_2. At pH values below 7.0, the medium was changed. This was done by sedimenting the cell aggregates for 45 min at $1 \times g$, aspirating the supernatant and adding fresh medium.

Determination of the cell number

For cell number estimation, washed cells were exposed to 0.2% (v/v) Triton X-100 dissolved in phosphate-buffered saline. After solubilization of the plasma membranes, the remaining (nonsolubilized) nuclei were counted with a hemocytometer or with a Coulter counter.

Preparation of plasma membrane vesicles

For the preparation of plasma membrane vesicles 1–3 l of spinner culture with a maximum density of $3 \cdot 10^7$ cells/ml were used. 1 day before harvesting the PC-12 cells, reserpine (10 μM, dissolved in dimethylsulfoxide, final concentration 0.1%) was added to deplete the store of endogenous catecholamines [8] and to inhibit the granular amine-carrier. Cells were sedimented for 45 min (at $1 \times g$) and then washed twice in phosphate-buffered saline. After determination of the wet weight (all following steps refer to it), cells were suspended in 'lysis buffer' of the following composition: 10 mM Tris-HCl (pH 7.4)/2 mM $MgCl_2$/1 mM dithiothreitol/1 mM PMSF. After incubation for 10 min (at 0 °C), cells were broken with ten strokes of a Dounce homogenizer (Wheaton, 7 ml, tight-fitting pestle). The homogenate was diluted 8–10-fold in 'dilution buffer' (140 mM NaCl/10 mM Tris-HCl (pH 7.4)/2 mM $MgCl_2$/0.4 mM PMSF/0.1% mercaptoethanol) and centrifuged for 15 min at $50 \times g$ (4 °C, swing-out rotor). The pellet was resuspended in 10 ml dilution buffer/ g wet wt. Both supernatant and resuspended pellet were spun again for 5 min at $1000 \times g$. The resulting supernatants were discarded; the pellets were suspended in either 3 ml EDTA buffer/g wet wt, (pellet of the $50 \times g$ supernatant) or 10 ml EDTA buffer/g wet wt, (resuspended $50 \times g$ pellet). The EDTA buffer consisted of 140 mM NaCl/10 mM Tris-HCl (pH 7.4)/2 mM Na_2EDTA/1 mM $CaCl_2$/0.1% mercaptoethanol. The diluted fractions were layered on top of a linear sucrose gradient (20–56% sucrose (w/v)/135 mM potassium phosphate (pH 7.4)/1 mM Na_2EDTA/0.3 mM $CaCl_2$).

For sucrose density gradient centrifugation, two rotors were used: a TST 28.35 swing out rotor (Kontron) with 35 ml buckets and a TFT 45.94 fixed angle rotor (Kontron) with 94 ml centrifuge tubes for higher capacity (10 ml membrane suspension instead of 3 ml for the swing out rotor). The 94 ml centrifuge tubes were rinsed with a silicon solution (to prevent biological material from sticking to the wall of the centrifuge tubes) and dried at ambient temperature. The gradients were spun for 2 h at 25 000 rpm (TST 28.35) or 1.5 h at 35 000 rpm (TFT 45.94) and fractionated with an ISCO UA-5 UV monitor (equipped with a flow cell) by underlaying a 65% sucrose solution to the gradient. To fractionate the 94 ml gradients of the fixed angle rotor a special adapter was constructed to hold the centrifuge tubes.

The plasma membrane peak (see Results) was diluted 1 : 8 in 'potassium phosphate buffer' (135 mM potassium phosphate (pH 7.4)/1 mM $MgCl_2$/1 mM ascorbic acid/1 mM dithiothreitol)

and pelleted (2 h, 35000 rpm, TFT 45.94 rotor). The membranes were suspended either in 2.5 ml potassium phosphate buffer/g wet wt, ('nucleosomal' membranes) or in 1.25 ml potassium phosphate buffer/g wet wt. ('microsomal' membranes). The final protein concentration was in the range between 0.4 and 0.8 mg/ml. All operations were carried out at 4°C; the membrane suspensions were stored in liquid nitrogen until use.

Enzyme assays

(a) ($Na^+ + K^+$)-ATPase (EC 3.6.1.3). The assay of this marker enzyme for plasma membranes consisted of two parts: the hydrolysis reaction, which was carried out as described by Trumble et al. [9] and the determination of the liberated phosphate [10]. Nonspecific ATPase activity was evaluated in the presence of 1 mM ouabain in a parallel assay [11].

(b) Phosphodiesterase (EC 3.1.4.1). To measure the activity of this marker enzyme for plasma membranes, 25 µl of the fraction under study were mixed with 900 µl of a solution of bis(*p*-nitrophenyl)phosphate (Ca^{2+} salt, 0.33%, dissolved in 0.2 M Tris-HCl, pH 7.4). After incubation for 15 min at 37°C, the reaction tubes were placed on ice (for about 5 min) and then centrifuged at 10000 × *g* (for 2 min). From the supernatant, the absorbance of liberated *p*-nitrophenol [12] was measured at 405 nm.

(c) NADH dehydrogenase (EC 1.6.99.3). The activity of this marker enzyme for endoplasmic reticulum was determined as described by Avruch and Wallach [13].

(d) Succinate dehydrogenase (EC 1.3.99.1). The activity of this mitochondrial enzyme was measured with *p*-iodonitrotetrazolium violet as substrate [14].

Determination of the intracellular and intravesicular water space

The method is a modification of the method described by Werdan et al. [15].

(a) Intracellular water space. About $5 \cdot 10^7$ cells were washed twice in phosphate-buffered saline (50 × *g*, 5 min, 4°C). The final pellet was suspended in 600 µl ^{3}HOH solution (135 mM NaCl/5 mM KCl/10 mM Tris-HCl (pH 7.4)/1 mM dithiothreitol/1 mM $MgSO_4$/0.1 mCi ^{3}HOH) and incubated for 30 min at 37°C and thereafter for 1 h at 0°C. The cell suspension was then mixed (1 : 1) with a ^{3}HOH solution (see above) which additionally contained 0.5 mg/ml sorbitol and 14.4 µCi [^{14}C]sorbitol (as extracellular space marker). A 210 µl portion of the mixture was layered on a 40 µl cushion of silicone oil (1 part AR 20, 2 parts AR 200) in a Beckman microfuge tube (0.4 ml content). The tube was placed in a water-filled bucket of a TST 28.35 swing out rotor; the cap of the tube remained 2–3 mm above the water. After centrifugation (25000 rpm, 1 h, 4°C) the tube was frozen at −80°C and the tip of the tube was thereafter cut off. The radioactivity of the pellet was extracted overnight by shaking in scintillation solution (Beckman HP/b). From the ^{3}H and ^{14}C radioactivities of the supernatant and the pellet, the intracellular ^{3}HOH space of PC-12 cells was estimated.

(b) Intravesicular water space of plasma membrane vesicles. 6 ml (i.e., about 3 mg protein) of the thawed plasma membrane suspension (nucleosomal membranes) were spun at 100000 × *g* for 1 h. The membrane pellet was resuspended in 750 µl ^{3}HOH solution (see above) and incubated overnight at 4°C. The further procedure was identical with that described for whole cells, with the exception of the silicone oil mixture, which for this purpose consisted of 550 µl AR 20 plus 450 µl AR 200.

Electron microscopy

For scanning electron microscopy, samples of cells and plasma membrane vesicles were prepared as described by Hiller et al. [16]. The samples were examined with an ISI Super III A scanning electron microscope. For transmission electron microscopy, samples were prepared as described by Moss et al. [17]. Thin sections of the Epon resin were made with an Om U 3 (Reichert, F.R.G.) and placed on copper grids (200 mesh). The samples were incubated with uranyl acetate (4% in 70% methanol), contrasted in lead citrate (2% in water) and analysed in a Zeiss EM 10 transmission electron microscope.

Membrane labelling with ^{3}H-labelled concanavalin A

PC-12 cells (500 mg wet wt.) were incubated for 1 h at 0°C with 3.15 µCi ^{3}H-labelled concanavalin

A (25.2 Ci/mmol) in 0.5 ml phosphate-buffered saline; the plasma membranes were prepared thereafter as described above.

Protein assay

Proteins were determined as described by Ohnishi and Barr [18], i.e., by a combination of the methods described by Lowry et al. [19] and Weichselbaum [20].

Uptake and accumulation of [^{3}H]noradrenaline in membrane vesicles

Thawed nucleosomal membranes (18 μl) were mixed at 0 °C in an Eppendorf reaction tube (1.5 ml) with 2 μl of potassium-phosphate buffer, which contained, if desired, 10 μM desipramine. When the samples had reached room temperature (after about 10 min), the accumulation of [^{3}H]noradrenaline was started by the addition of 200 μl prewarmed (usually 31 °C) 'Na^+/Tris buffer'containing [^{3}H]noradrenaline (55 nM, 22.7 Ci/mmol) with or without 1 μM desipramine. The Na^+/Tris buffer consisted of 140 mM NaCl/10 mM Tris-HCl (pH 7.4)/1 mM $MgCl_2$/1 mM dithiothreitol/1 mM ascorbic acid. At the end of the incubation period, 200 μl of the mixture were withdrawn, mixed with 2 ml of an ice-cold 150 mM NaCl solution, and then immediately filtered through a Whatman GF/F filter. The filters were washed twice with 2 ml of physiological NaCl solution.

The filtration was performed in a special apparatus, which had the following advantages (compared with commercial system): (i) a small filtration area (about 7 mm in diameter) ensured a low background and enabled the use of very small amounts of membranes, and (ii) each sample could be exposed to the vacuum for exactly the same time by means of separate stopcocks for each filter.

The radioactivity remaining on the filter was extracted overnight by shaking in 3 ml scintillation cocktail.

Materials

The following drugs and substances were used. Bis (*p*-nitrophenyl)phosphate, Ca^{2+} salt (Riedel de Haen, Hannover); dithiothreitol, monensin (Calbiochem, Frankfurt); desipramine hydrochloride, reserpine (Ciba Geigy, Basel); (−)-noradrenaline hydrochloride (Hoechst, Frankfurt); NADH (Boehringer, Mannheim); silicone oil (Wacker Chemie, München); sucrose (Roth, Karlsruhe); Epon (Ladd Research, Burlington).

All other reagents were of purest available quality and purchased from Sigma (München), Serva (Heidelberg) or Merck (Darmstadt). (−)[7(*n*)-^{3}H]noradrenaline, D[^{14}C]sorbitol, [*acetyl*-^{3}H]concanavalin A and tritiated water were obtained from New England Nuclear (Dreieich, F.R.G.).

Results

(1) Isolation of plasma membranes

When PC-12 cells were incubated in the hypotonic lysis buffer, no significant lysis occurred, although the cells showed a considerable swelling. However, mild homogenization of these swollen cells with ten strokes of a tight-fitting Dounce homogenizer led to rupture of these cells (without damage of the nuclei). Mg^{2+} in the lysis buffer was important to keep the nuclei intact, since, in their absence, the homogenate formed considerable clusters (which obviously were caused by DNA leaking from nuclei).

After dilution of the homogenate in buffer with a physiological salt concentration (dilution buffer), low-speed centrifugation ($50 \times g$, 15 min) resulted in a pellet containing nuclei, some unbroken cells and aggregates of plasma membranes. High-speed centrifugation ($1000 \times g$, 5 min) of the supernatant yielded additional plasma membranes in the pellet. Since this pellet was strongly contaminated by mitochondria, this membrane fraction was designated as 'microsomes'. Resuspension of the $50 \times g$ pellet and high-speed centrifugation ($1000 \times g$, 5 min) resulted in a pellet which contained plasma membranes contaminated with nuclei; this fraction was, therefore, designated as 'nucleosomes'.

Both membrane fractions were suspended in buffer containing 2 mM EDTA (to complex Mg^{2+}, which impairs a clear separation of mitochondria from membranes) and 1 mM Ca^{2+} (to inhibit lysis of the nuclei) and layered on top of a linear sucrose gradient (containing 0.3 mM $CaCl_2$).

Centrifugation of the nucleosomal fraction with a swing out rotor or a high capacity fixed angle

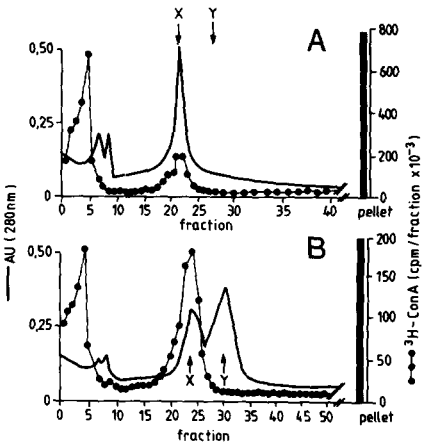

Fig. 1. Distribution of ultraviolet absorbance and ^{3}H-labelled concanavalin A radioactivity within a linear sucrose density gradient of the nucleosomal (A) and the microsomal (B) fraction. PC-12 cells were first labelled with ^{3}H-labelled concanavalin A (^{3}H-Con A) and thereafter homogenized as described in Materials and Methods. The pellets of the second differential centrifugation (nucleosomes and microsomes) were layered on top of a linear sucrose density gradient (20–56%) and spun for 2 h at 25000 rpm in a swing out rotor (Kontron TST 28.25). The gradients were then fractionated (625 μl per fraction) as described in Materials and Methods. Shown is the absorbance at 280 nm (in arbitrary units, AU) and the radioactivity (in cpm) per fraction. The bar on the right side of the figure shows the radioactivity found in the pellet of the gradient. X = maximum of [^{3}H]Con A radioactivity, which coincided with a maximum in the activity of ($Na^+ + K^+$)-ATPase and phosphodiesterase (Table I); Y = maximum of the succinate dehydrogenase and NADH dehydrogenase activities (Table I).

rotor resulted in a single visible band at a density of 1.120 g/cm^3 and a large pellet (containing nuclei and some unbroken cells); whereas, the microsomal fraction yielded two visible bands at a density of 1.120 g/cm^3 and 1.135 g/cm^3 and a small pellet. After labelling of intact PC-12 cells with ^{3}H-labelled concanavalin A, the peak of the ^{3}H radioactivity comigrated with the single band in the first gradient and the lower-density band of the second gradient (Fig. 1). This indicates an enrichment of plasma membranes in these bands. The more dense material (second band) consisted of mitochondria and membranes of the endoplasmic reticulum (as judged by the relative activities of the corresponding marker enzymes; Table I).

The sucrose-containing plasma membrane fractions were separated and washed in potassium phosphate buffer. Resuspension of the pellet in the same buffer resulted in the final fractions of plasma membrane vesicles which were used for further experiments. Recovery of protein in this fraction was about 1.7%.

(2) Properties of the plasma membrane vesicle fraction

The relative activities (in comparison to the cell homogenate) of marker enzymes of the different fractions of the plasma membrane preparation are shown in Table I. The plasma membrane fractions

TABLE I

MARKER ENZYME ACTIVITIES

Activities (related to those of the homogenate) of the marker enzymes for plasma membranes (($Na^+ + K^+$)-ATPase and phosphodiesterase (PDE)), mitochondria (succinate dehydrogenase (SDH)) and endoplasmic reticulum (NADH dehydrogenase) within fractions obtained during the isolation of plasma membrane vesicles. The letters X and Y (in parentheses) refer to the fractions with a high activity of either ($Na^+ + K^+$)-ATPase (X) or succinate dehydrogenase (Y) shown in Fig. 1. The mean protein recovery of the two ($Na^+ + K^+$)-ATPase containing fractions was about 1.7%. Shown are means ± S.E. of 3–6 preparations.

Fraction	Relative activity			
	($Na^+ + K^+$)-ATPase	PDE	SDH	NADH dehydrogenase
Homogenate	1	1	1	1
Nucleosomal membranes	23.7 ± 5.6 *	8.4 ± 4.9	0.6 ± 0.3	0.9 ± 0.3
Microsomal membranes (X)	23.9 ± 10.1 *	5.3 ± 0.9	1.7 ± 0.7	1.1 ± 0.2
Microsomal membranes (Y)	1.6 ± 0.5 *	0.3 ± 0.1	18.5 ± 6.3	10.1 ± 1.8

* For measurements of ($Na^+ + K^+$)-ATPase activity, Na^+/Tris buffer (instead of potassium phosphate buffer) was used for the final wash of the plasma membranes.

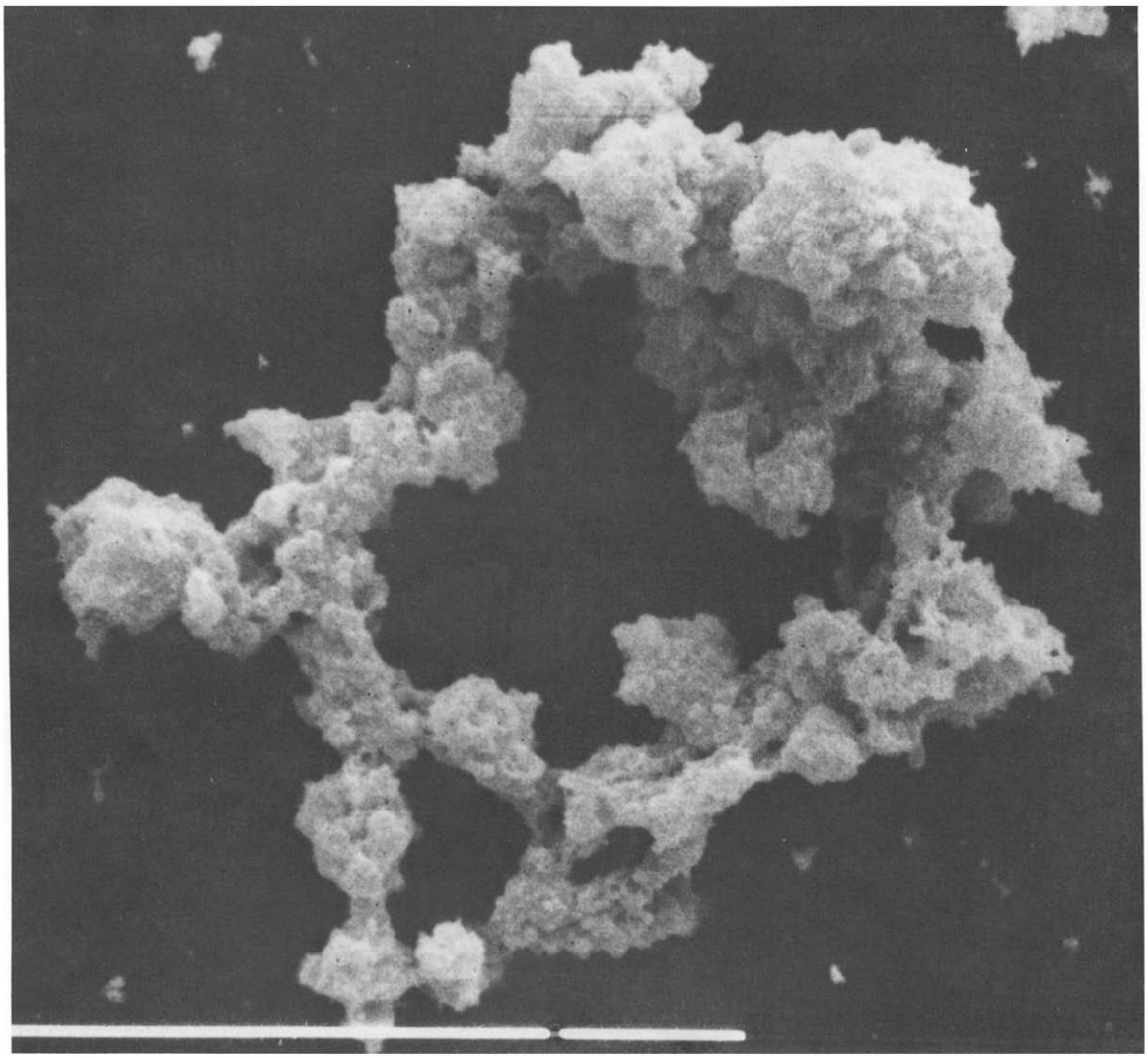

Fig. 2. Scanning electron micrograph of plasma membrane vesicles. Right bar = 5 μm; further details see Materials and Methods.

showed about 24-fold enrichment in $(Na^+ + K^+)$-ATPase activity and 5–8-fold enrichment in phosphodiesterase activity (both are marker enzymes of plasma membranes). The activities of succinate dehydrogenase and NADH dehydrogenase (as marker enzymes for mitochondria and endoplasmic reticulum, respectively) were decreased in these fractions (Table I).

Morphological analysis of the plasma membrane fraction by scanning electron microscopy indicates a vesicular structure; the vesicles form aggregates of up to cell-sized diameter (Fig. 2). Transmission electron micrographs of a thin section of plasma membranes also indicated vesicular structures (not shown).

For the plasma membrane vesicles, an intravesicular volume of 1.84 μl/mg protein was determined by means of tritiated water and [^{14}C]sorbitol (see Materials and Methods). The accuracy of this method was checked with intact PC-12 cells. The intracellular space of PC-12 cells was 2.40 μl/mg protein. A very similar value (2.14 μl/mg protein; 1 mg protein corresponds to $9.8 \cdot 10^6$ cells) was estimated from electron micrographs (assuming a spherical shape of the cells).

(3) Transport of [^{3}H]noradrenaline

For the demonstration of a carrier-mediated transport of noradrenaline, plasma membrane vesicles (isolated from the nucleosomal fraction) were diluted in Na^+/Tris buffer containing [^{3}H]noradrenaline (final concentration 50 nM) and incubated for 1–12 min (at 0, 18, 25 and 37 °C). The intravesicular accumulation of [^{3}H]noradrenaline showed a time- and temperature-dependence (Fig. 3). Maximum accumulation of [^{3}H]noradrenaline was achieved after 6 min at 31 °C. The initial accumulation (after 1 min) increased with increasing temperature (Fig. 3). At 31 and 37 °C, the accumulation declined after having reached a maximum (after 6 and 2 min, respectively).

The uptake and accumulation was inhibited by 1 μM desipramine (Fig. 3) and at 0 °C (not shown). The remaining component of uptake was of about the same magnitude under both conditions.

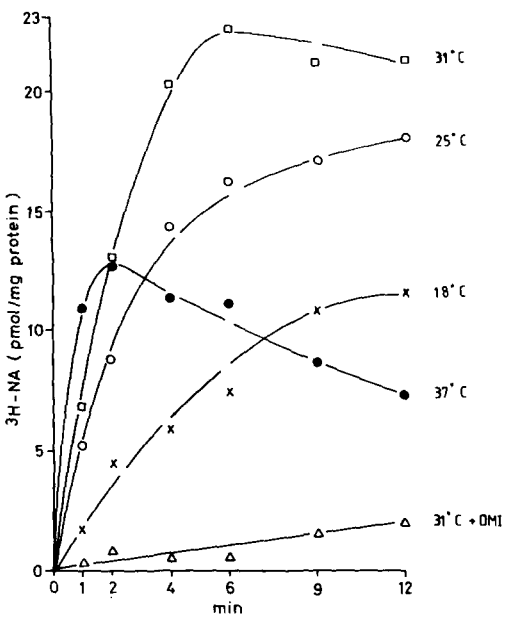

Fig. 3. Time- and temperature-dependent accumulation of [^{3}H]noradrenaline (^{3}H-NA) in plasma membrane vesicles of PC-12 cells. Membrane vesicles (about 14 μg protein) equilibrated with potassium phosphate buffer were diluted into Na^+/Tris-buffer containing 50 nM [^{3}H]noradrenaline and in one experimental group additionally 1 μM desipramine (DMI), incubated at 0, 18, 25, 31 and 37 °C for 1–12 min, filtered and counted as described under Materials and Methods. Shown are mean values of the accumulation of [^{3}H]noradrenaline (after subtraction of the accumulation observed at 0 °C) of a typical experiment performed in triplicates.

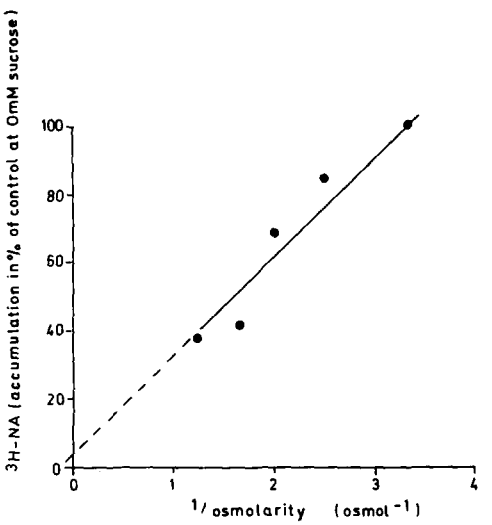

Fig. 4. Osmotically sensitive accumulation of [^{3}H]noradrenaline (^{3}H-NA) in plasma membrane vesicles of PC-12 cells. 50 μl of the plasma membrane suspension were incubated for 10 min (at 0 °C) with the same volume of potassium phosphate buffer (which contained 0, 200, 400, 600 or 1000 mM sucrose, each with or without 2 μM desipramine). Then 1000 μl of prewarmed incubation solution (55 nM [^{3}H]noradrenaline and 0, 100, 200, 300 or 500 mM sucrose in Na^+/Tris-buffer with and without 1 μM desipramine) was added. After 2 min at 31 °C, the accumulation of [^{3}H]noradrenaline was stopped. The figure shows mean values of an experiment performed in triplicates of the desipramine-sensitive [^{3}H]noradrenaline accumulation plotted against the reciprocal value of the osmolarity of the incubation solution.

The accumulation of [^{3}H]noradrenaline (determined after 1 min at 31 °C) increased linearly with increasing membrane protein concentration in the incubation solution (at least up to 1 mg protein/ml; data not shown). The accumulation of [^{3}H]noradrenaline (after 2 min at 31 °C) was sensitive to changes of the osmotic pressures of the incubation solution. Fig. 4 shows that the accumulation decreases with increasing osmolarity (caused by an addition of sucrose); extrapolation of osmolarity to infinitely high values results in nearly no accumulation.

Discussion

Preparation of plasma membrane vesicles

Studies using isolated membrane vesicles have significantly contributed to our knowledge on

molecular mechanisms of the transport of molecules across cell membranes (for review see Lever [21]).

Preparation of plasma membrane vesicles for transport studies requires first a large amount of an homogeneous population of cells and second a method which makes possible the isolation of purified sealed, osmotically sensitive membrane vesicles with a functional transport carrier. The first criterion was fulfilled in this study by the use of a clonal cell line. PC-12 cells, which normally grow as monolayers on tissue culture dishes, were adapted to grow in spinner culture. This adaption of culture conditions obviously did not cause a loss of transport activity. Recognizable differences to PC-12 cells grown in monolayer culture were a reduction of the cell-generating time and, to a minor extent, also of the size of the cells, whereas the tendency of PC-12 cells to adhere to one another and to form clumps remained unchanged. The last feature was advantageous for harvesting the cells in spinner culture by sedimentation at $1 \times g$. The procedure described here to obtain membrane vesicles is based on osmotic swelling of the cells by incubation in a hypotonic salt solution (without lysis) followed by a mild and controlled homogenization with a tight-fitting Dounce homogenizer. When a Polytron homogenizer, nitrogen cavitation or a sonifier was used for cell rupture, the resulting membrane fraction was less pure, the yield was reduced and the sucrose density gradient centrifugation did not result in a sharp, distinct band.

To prevent lysis of nuclei, Mg^{2+} was included in the lysis buffer [22]. Obviously due to the characteristic tendency of PC-12 cells to stick together, the separated cytoplasmic membrane fragments form large aggregates which, by differential centrifugation, sediment together with nuclei (nucleosomal fraction).

The distribution of ^3H-labelled concanavalin A within the linear sucrose gradient showed two peaks, one associated with plasma membranes, the other associated with the pellet, where the ^3H activity was bound to either intact cells or to membranes sticking to nuclei or whole cells.

Linear sucrose gradient centrifugation of the microsomal fraction also revealed a plasma membrane fraction. However, in contrast to the plasma membrane fraction isolated from the nucleosomal fraction membranes were slightly contaminated with mitochondrial membranes which banded at a slightly higher sucrose density. Nevertheless, the plasma membranes derived from the microsomal fraction could also be used for transport studies (no data shown). In both cases, the final membrane fraction, after washing in potassium phosphate buffer, obviously consisted of sealed plasma membrane vesicles. Nonvesicular membrane fragments are less dense than sealed plasma membrane vesicles and mitochondrial membranes [23]. Plasma membranes of the microsomal fraction appeared at a density of 1.120 g/cm^3, i.e., in the range where sealed plasma membranes prepared from different cell sources were found [23]; whereas, mitochondrial membranes and membranes derived from endoplasmic reticulum appeared at a density of 1.135 g/cm^3. Independent evidence for the vesicular nature of the isolated plasma membrane fraction comes from electron microscopic pictures which clearly show vesicles with a mean diameter of roughly 0.3 μm, a size which seems to be typical for plasma membrane vesicles derived from isolated cells by mild shear forces [22]. The isolated membrane vesicles are osmotically sensitive, since the [^3H]noradrenaline accumulation in the vesicles (after 2 min) was a linear function of the osmolarity of the incubation medium, extrapolating to about zero accumulation at infinitely high osmolarity (for further discussion of [^3H]noradrenaline transport, see below). In isosmotic solutions, the vesicular volume (1.84 μl/mg protein) was within the range of 1–4 μl/mg membrane protein estimated for different animal cell vesicle preparations used in transport studies [21,24,25].

The degree of purification of the isolated membrane vesicle fraction becomes evident from the enrichment of the activities of the plasma membrane marker enzymes phosphodiesterase and $(Na^+ + K^+)$-ATPase. While the activity of the latter enzyme was 24-fold increased (relative to the homogenate), phosphodiesterase activity was enriched only about 8-fold (Table I). This discrepancy is obviously due to the fact that phosphodiesterase is not as specific a plasma membrane marker enzyme as $(Na^+ + K^+)$-ATPase is. Indeed, it has been shown that significant phosphodiesterase activity is found in the soluble frac-

tion of bovine adrenal medullary cells [26]. From the 24-fold enrichment of $(Na^+ + K^+)$-ATPase activity in the plasma membrane fraction (derived both from nucleosomal and microsomal fractions) and from protein recovery of 1.7%, a total yield of about 40% of plasma membranes in the combined final membrane fraction can be estimated. Additional evidence for purification of the plasma membranes is the coincidence of maximal $(Na^+ + K^+)$-ATPase activity and maximal ^{3}H-labelled concanavalin A radioactivity within the fractions of the sucrose gradient. Since, in sucrose medium, a partial dissociation of the bound lectin was observed, the distribution of bound ^{3}H-labelled concanavalin A radioactivity in the homogenate of PC-12 cells was also checked using an isosmotic Percoll density gradient. For this purpose the nucleosomal fraction was layered on a preformed percoll gradient (27% (v/v) Percoll in 140 mM NaCl/10 mM Tris-HCl (pH 7.4)/1 mM dithiothreitol/2 mM Na_2EDTA was centrifuged for 40 min at $40\,000 \times g$). After centrifugation (for 30 min at $3600 \times g$) nearly 80% of the radioactivity were recovered from the plasma membrane peak (at a density of 1.025 g/cm^3); the residual radioactivity was associated with a lower band (at a density of 1.045 g/cm^3) which contained nuclei and intact cells. With Percoll, basically the same degree of purification of plasma membranes (from the nucleosomal fraction) was obtained as with sucrose. However, Percoll material could not be removed completely from the plasma membranes and it bound [^{3}H]noradrenaline; therefore, this method was not further used.

The higher capacity and a shorter running time were the advantages of the large volume fixed angle rotor (TFT) 45.94) compared with commonly used swing out rotors (e.g., TST 28.35 in this study). To facilitate sucrose density gradient centrifugation in a fixed-angle rotor, the centrifuge tubes had to be siliconized to avoid sticking of plasma membranes to the wall of the tube.

Transport of -^{3}H]noradrenaline

In the presence of a sodium gradient across the membrane (out > in), the isolated membrane vesicles (loaded with potassium phosphate) take up and accumulate [^{3}H]noradrenaline. Beside its dependence on a sodium (and potassium) gradient, the uptake (and accumulation) of [^{3}H]noradrenaline was sensitive to osmotic pressure, reduced by a decrease of the temperature (at 0 °C no uptake took place) and inhibited by the antidepressant desipramine (a typical inhibitor of neuronal noradrenaline uptake).

The decrease of accumulation of [^{3}H]noradrenaline by an increase of the osmotic pressure is an evidence that [^{3}H]noradrenaline was not bound to the membrane but accumulated (after its transport) within an osmotically sensitive space. From the internal volume (1.84 µl/mg protein) of the membrane vesicles and from the accumulation observed after 6 min incubation at 31 °C with 50 nM [^{3}H]noradrenaline (Fig. 1), an about 550-fold enrichment of [^{3}H]noradrenaline within the membrane vesicles can be estimated. A similar high degree of accumulation (about 500-fold) was described for 5-hydroxytryptamine in plasma membrane vesicles isolated from blood platelets [27]. The decline with time of the accumulation of [^{3}H]noradrenaline (especially at temperatures above 25 °C) is obviously the result of a slow collapse of the artificially imposed Na^+ and/or K^+ gradient, which cannot be maintained in the absence of ATP, i.e., in the absence of an active $(Na^+ + K^+)$-ATPase.

The easy and high-yield preparation of PC-12 plasma membranes offers a good basis for the further molecular characterization (and final isolation) of the noradrenaline transport system of PC-12 cells.

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References

1 Greene, L.A. and Tischler, A.S. (1982) Adv. Cell. Neurobiol. 3, 373–414
2 Greene, L.A. and Rein, G. (1977) Brain Res. 129, 247–263

3 Iversen, L.L. (1963) Br. J. Pharmacol. 21, 523–537
4 Morgan, J.G. (1976) Neuroscience 1, 159–165
5 Wheeler, D.D. (1978) J. Neurochem. 30, 109–120
6 Harder, R. and Bönisch, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 312, Suppl. R46
7 Greene, L.A. and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2424–2428
8 Schubert, D. and Klier, F.G. (1977) Proc. Natl. Acad. Sci. USA 74, 5184–5188
9 Trumble, W.R., Slutko, J.L. and Reeves, P. (1980) Life Sci. 27, 207–214
10 Ames, B.N. (1966) Methods Enzymol. 8, 115–118
11 Matsui, H. and Schwartz, A. (1968) Biochim. Biophys. Acta 151, 655–663
12 Koerner, J.F. and Sinsheimer, R.L. (1957) J. Biol. Chem. 228, 1049–1053
13 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334–350
14 Porteons, J.W. and Clark, B. (1965) Biochem. J. 96, 159–171
15 Werdan, K., Lehner, K., Cremer, T., Stevenson, F.G. and Messerschmidt, O. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 91–104
16 Hiller, G., Weber, K., Schneider, L., Parajsz, C. and Jungwirth, C. (1979) Virology 98, 142–153
17 Moss, B., Rosenblum, E.N. and Grimley, P.M. (1971) Virology 45, 123–134
18 Ohnishi, S.T. and Barr, J.K. (1978) Anal. Biochem. 86, 193–200
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Weichselbaum, T.E. (1946) Amr. J. Clin. Res. 16, 40–49
21 Lever, J.E. (1980) CRC Crit. Rev. Biochem. 7, 187–246
22 Wallach, D.F.H. (1976) J. Cell. Physiol. 89, 769–770
23 Neville, D.M., Jr. (1976) in Biochemical Analysis of Membranes (Maddy, A.H., ed.), pp. 27–54, Chapman & Hall, London
24 Quinlan, D.C., Parnes, J.R., Shaolm, R., Garvey, T.Q., Isselbacher, K.J. and Hochstadt, J. (1976) Proc. Natl. Acad. Sci. USA 73, 1631–1635
25 Lopez Solis, R.O. and Durham, J.P. (1983) Biochim. Biophys. Acta 729, 237–248
26 Tirrell, J.G. and Coffee, C.J. (1983) Arch. Biochem. Biophys. 222, 380–388
27 Rudnick, G. and Nelson, P.J. (1978) Biochemistry 17, 5300–5303