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PREPARATION OF PLASMA MEMBRANES FROM ISOLATED CELLS OF NEWBORN RAT BRAIN

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SUMMARY

A procedure is presented for the isolation of plasma membranes from a suspension of immature brain cells. The cells were homogenized in a hypotonic medium and plasma membranes and other subcellular fractions were separated by differential centrifugation in sucrose. The recovery of protein in the plasma membrane fraction was 1.3%.

1. Electron microscopy showed that plasma membranes were long, mainly open membrane strands. No nerve endings, myelin or mitochondria could be detected in the fraction.

2. The concentrations of protein, phospholipid and RNA were recorded in the subcellular fractions. Phospholipid was concentrated in plasma membranes 2.5 times above the level in the homogenate. By contrast, the RNA content in the fraction was low.

3. The activities of $(Na^+–K^+)$-activated ATPase, K^+-stimulated phosphatase and 5′-nucleotidase were concentrated 7.1, 3.1 and 3.6 times, respectively, in the plasma membrane fraction compared to the activities in the homogenate. These results suggest that even in the brain $(Na^+–K^+)$-activated ATPase is mainly localized in the plasma membrane.

INTRODUCTION

The integrative function of the brain depends on the activity of excitable membranes. The isolation of two membrane structures specific to the nervous system, synaptosomes and myelin[1,2], has greatly contributed to our present understanding of the biochemical basis of nervous activity. Yet the complexity of the adult brain in regard to cell types and membrane structures, including synaptosomes, myelin, axons and dendrites, has seriously impeded attempts to isolate membrane elements existing in small amounts in brain tissue, for instance plasma membranes. Specialized areas of the plasma membrane, the postsynaptic membranes, are thought to be primarily involved in the postsynaptic events of transmission. There has been much speculation about the involvement of the plasma membrane in chemical transmission, but quantitative data are lacking because of difficulties in isolation. So far, preparations have been described only for the axolemma from squid nerves[3,4]

synaptosomal plasma membranes[5,6] and recently for isolated rabbit neuronal soma[7].

In this paper we present a procedure for the isolation of plasma membranes from a suspension of immature brain cells. The use of newborn rat brain as starting material has several advantages over adult tissue in regard to the isolation of plasma membranes. The newborn rat brain is devoid of myelin, poor in synaptosomes and intracellular membranes[8] simplifying the isolation of plasma membranes. Moreover, it contains no glia but neurons, neuroblasts and a few spongioblasts[8], and thus forms a good source for the enrichment of membranes of neuronal origin. Isolated cells rather than whole brain were used to provide material for *in vitro* studies.

Owing to the evident difficulties of isolating plasma membranes from brain no special markers are available. Instead, data from other tissues on the molecular composition, enzymatic characteristics and electron microscopy of various membrane structures are used for identification of the fractions prepared.

MATERIALS AND METHODS

Isolation and homogenization of cells

Preparation of cells from newborn rat brain has been described previously[9]. Cortices from 10 to 15 rats (3–6 days old) were torn in small pieces with two forceps in 50 ml of cold dispersion medium containing 0.1% trypsin, 1 mM EDTA, 280 mM sucrose, 10 mM glucose, 20 mM sodium phosphate and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid buffer (pH 7.5). The suspension was shaken at 37 °C under air and at 0, 1 and 5 min it was gently sucked 3 times in a large pipette to help the dispersion of cells. At 30 min 2 vol. of Eagle's minimal essential medium were added and undissociated particles were sedimented by gravity for 2 min. The supernatant was decanted and centrifuged at $300 \times g$ for 10 min; the resulting cellular pellet was suspended in calf serum. The undissociated particles were suspended in 25 ml of the dispersion medium and cells were isolated as described above. The two fractions of cells in serum were combined and centrifuged at $300 \times g$ for 10 min followed by two additional washings with the dispersion medium.

The washed cellular pellet was suspended with a pipette in 30 ml of 90 mM sucrose, 1 mM $MgCl_2$ and 40 mM Tris buffer (pH 7.6). In this hypotonic medium cells were left on ice for 5–10 min and then homogenized with a loosely fitting Teflon-glass homogenizer, using 15 strokes. The homogenate was filtered rapidly through a nylon mesh (hole size 30 μm) under a vacuum and 1.0 M sucrose was added to adjust the concentration of sucrose to 0.2 M.

Isolation of subcellular fractions

The fractionation scheme is shown in Fig. 1. The suspension obtained was centrifuged at $3500 \times g$ for 5 min. The pellet was resuspended in 20 ml of 2.2 M sucrose containing 1 mM $MgCl_2$ and transferred to a centrifuge tube with 5 ml layers of 1.3 M and 0.25 M sucrose. The fractions were separated by centrifuging in a Spinco SW-25 rotor at $63000 \times g$ for 60 min. The pellet contained nuclei, the 2.2/1.3 M sucrose interphase mainly large sheets of rough membranes and the 1.3/0.25 M sucrose interphase cell processes.

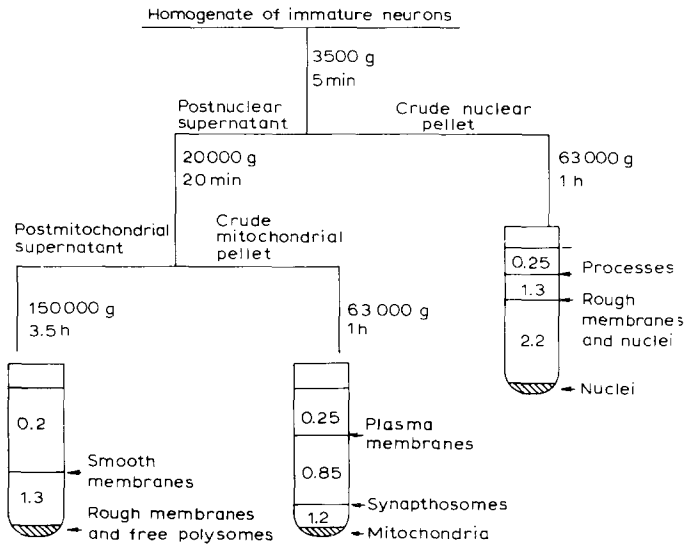

Fig. 1. A flow sheet for the isolation of membrane fractions from immature neurons.

The postnuclear supernatant was centrifuged at $20000 \times g$ for 20 min. The supernatant was made 15 mM with CsCl, and smooth and rough endoplasmic reticulum were separated according to Dallner[10] by centrifuging in a Spinco Ti-50 rotor at $150000 \times g$ for 3.5 h. The $20000 \times g$ pellet was suspended in 0.25 M sucrose and sedimented at $20000 \times g$ for 20 min to remove small microsomal membranes. The resulting pellet was suspended in 30 ml of 0.85 M sucrose and divided in two. Each part was placed in a centrifuge tube containing a 5-ml layer of 1.2 M sucrose at the bottom and a 10-ml layer of 0.25 M sucrose on top. The tubes were spun in a SW-25 rotor at $63000 \times g$ for 60 min. The pellet contained mitochondria, the 1.2/0.85 M sucrose interphase some synaptosomes, and the 0.85/0.25 M sucrose interphase plasma membranes.

The separate fractions were collected with a Pasteur pipette. diluted with equal volumes of water and centrifuged to pellets to be suspended in 0.25 M sucrose for analysis. In figures and tables the fractions were for convenience called by the typical constituents found in these fractions by electron microscopic examination.

Determinations

Protein was measured according to Lowry *et al.*[11] and RNA according to Schneider[12]. Phospholipids were extracted by the Folch system[13] and determined by the method of Bartlett[14] as modified by Kankare and Suovaniemi[15].

Enzyme assays

ATPase (EC 3.6.1.3.) was assayed in a medium containing 20 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM KCl, 100 mM NaCl and 5 mM ATP as the Tris salt in a total volume of 250 μl containing 5–20 μg protein. The mixture was incubated at 37 °C for 5 min and the reaction was terminated by addition of 400 μl of 12% trichloroacetic acid. The solution was centrifuged and the P_i content of the supernatant was determined according to Chen *et al.*[16]. The increment of

activity on addition of Na^+ and K^+ was taken as the (Na^+–K^+)-activated ATPase activity.

K^+-stimulated phosphatase (EC 3.1.3.1.) was assayed in a medium containing 20 mM Tris–HCl buffer (pH 7.4), 10 mM KCl, 5 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate as the Tris salt. The incubation conditions were as above; the reaction was terminated by adding 800 μl 0.5 M NaOH. The mixture was centrifuged and the absorbance of the supernatant was measured at 410 nm.

5′-Nucleotidase (EC 3.1.3.5.) was determined according to Heppel and Hilmoe[17]. Cholinesterase (EC 3.1.1.8.) was determined by the method of Ellman *et al.*[18]. Acetylcholinesterase (EC 3.1.1.7) was assayed by using its specific inhibitor, 284C51 in the assay medium of cholinesterase at 0.01 mM concentration.

Electron microscopy

The pellets of subcellular fractions were fixed in 2.5% glutaraldehyde buffered to pH 7.2 with 0.2 M sodium phosphate and treated according to Sabatini *et al.*[19].

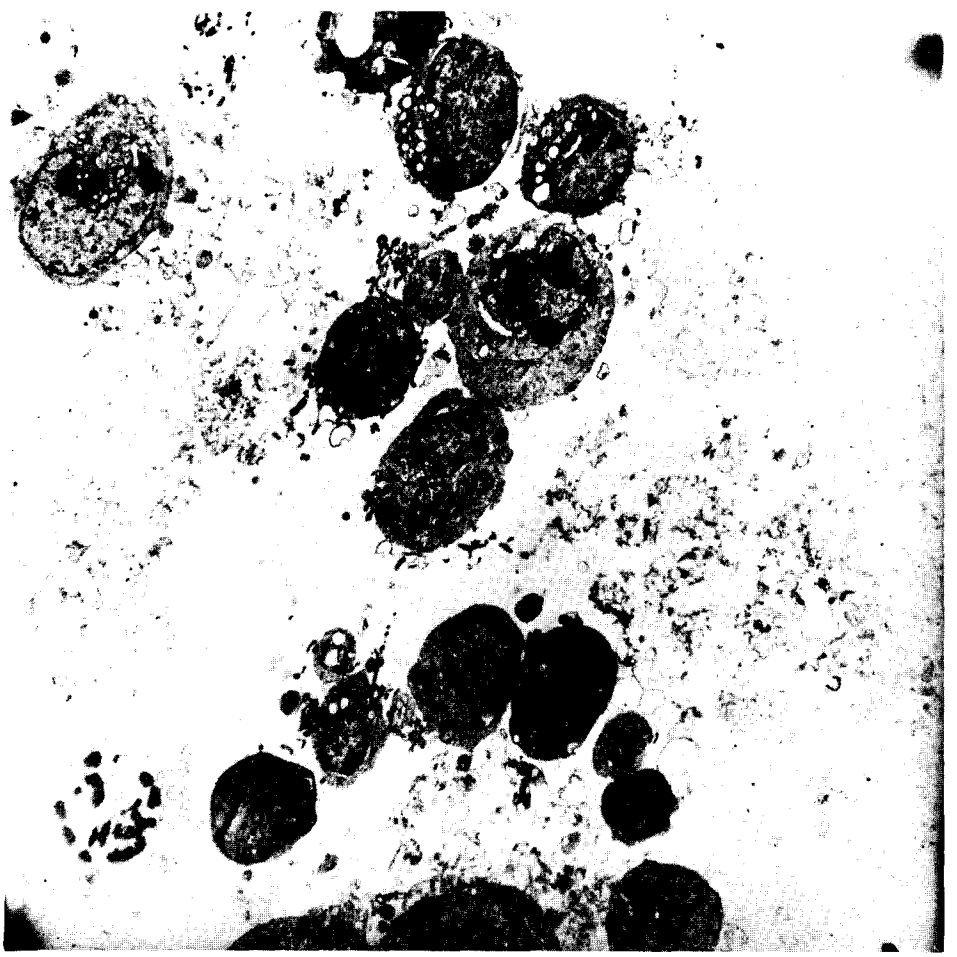

Fig. 2. A general electron microscopic view of the isolated neurons before homogenization. 1200 ×.

RESULTS

Electron microscopy of the fractions

A large electron microscopic field of the isolated cells is shown in Fig. 2. The preparation consists of cells retaining their morphological integrity and plasma membranes as described elsewhere[9]. Identification of undifferentiated cells is difficult, but developmental studies on the rat brain[8,20] suggest that the cells present shortly after birth are overwhelmingly of neuronal origin. Accordingly, this preparation has been referred to as immature neurons, although a small number of spongioblasts is obviously also present.

Fig. 3 shows the plasma membrane fraction isolated from immature neurons. Large membrane strands and a few small scattered particles, which are presumably

Fig. 3. An electron micrograph of the plasma membrane fraction isolated from immature neurons. 10000×.

remains of cytoplasmic material, can be seen. By contrast, no myelin, synaptosomes or mitochondria can be detected in the preparation.

The other subcellular fractions indicated in Fig. 1 were studied as well (not shown). The appearance of these samples was distinctly different from plasma membranes. Only the isolated cell processes contained some large membrane structures similar to those found in the plasma membrane fraction, but these presented a minor constituent as the fraction was rich in axon-like strands. The isolated nuclei and mitochondria were pure preparations, while the synaptosomal fractions were contaminated by mitochondria and small membrane vesicles. The isolated smooth endoplasmic reticulum was enriched in small vesicles, frequently darkly stained, and Golgi elements. Rough membranes were found in two separate fractions (Fig. 1).

Distribution of protein, phospholipid and RNA in the fractions

The protein, phospholipid and RNA contents of the fractions were determined (Table I). RNA was found to be markedly concentrated in the two rough membrane fractions, which contained almost 3 times as much RNA as the whole homogenate per unit protein. By contrast, the amounts of RNA in smooth membranes, plasma membranes and synaptosomes were found to be relatively low. Phospholipid as a membrane constituent was concentrated in the plasma membrane fraction 2.5 times, and in the smooth membranes 1.7 times above the level in the homogenate. The phospholipid content of the fraction containing nuclei and rough membranes is low. On a protein basis the yield of the present fractionation is 38%. The plasma membrane fraction contains 1.3% of the total protein.

TABLE I

DISTRIBUTION OF PROTEIN, PHOSPHOLIPID AND RNA IN SUBCELLULAR FRACTIONS ISOLATED FROM IMMATURE NEURONS

Each figure represents the means ± S.E. of 4–7 experiments.

	Protein (%)	*Protein/ phospholipid ratio*	*Protein/ RNA ratio*
Homogenate	100	3.3 ± 0.3	14.9 ± 1.7
Nuclei	3.8 ± 1.2	7.2 ± 1.4	10.6 ± 3.3
Rough membranes and nuclei	4.9 ± 1.7	4.0 ± 0.6	5.0 ± 2.0
Processes	2.7 ± 0.9	2.6 ± 0.1	19.3 ± 4.4
Mitochondria	12.1 ± 3.0	3.6 ± 0.2	27.0 ± 5.4
Synaptosomes	2.8 ± 1.0	2.5 ± 0.2	30.3 ± 6.2
Plasma membranes	1.3 ± 0.3	1.3 ± 0.3	30.2 ± 8.0
Rough membranes and free polysomes	3.4 ± 0.7	5.4 ± 0.8	5.9 ± 1.3
Smooth membranes	6.8 ± 1.2	1.9 ± 0.2	39.4 ± 5.6
Yield	37.8	—	—

TABLE II

RELATIVE ACTIVITIES OF ENZYMES IN SUBCELLULAR FRACTIONS ISOLATED FROM IMMATURE NEURONS AS COMPARED TO HOMOGENATE

Each figure represents the means ± S.E. of 3–7 experiments. The absolute specific activities of the enzymes in the homogenate were 0.022 μmole P_i/mg protein per min for (Na^+–K^+)-activated ATPase, 0.013 μmole nitrophenol/mg protein per min for K^+-stimulated phosphatase, 0.027 μmole P_i/mg protein per min for 5′-nucleotidase and 19.8 nmoles/mg protein per min for cholinesterase.

	(Na^+–K^+)-activated ATPase	*K^+-stimulated phosphatase*	*5′-Nucleotidase*	*Cholinesterase*
Homogenate	1.00	1.00	1.00	1.00
Nuclei	0.15 ± 0.10	0.38 ± 0.14	0.43 ± 0.15	0.40 ± 0.22
Rough membranes and nuclei	1.48 ± 0.18	1.20 ± 0.12	1.36 ± 0.25	1.40 ± 0.26
Processes	1.52 ± 0.42	1.13 ± 0.16	1.19 ± 0.17	1.44 ± 0.34
Mitochondria	1.67 ± 0.24	1.48 ± 0.32	0.96 ± 0.24	0.89 ± 0.25
Synaptosomes	1.00 ± 0.20	1.57 ± 0.42	1.29 ± 0.20	1.79 ± 0.59
Plasma membranes	7.13 ± 0.96	3.10 ± 0.31	3.57 ± 0.56	1.54 ± 0.50
Rough membranes and free polysomes	1.36 ± 0.37	0.72 ± 0.34	1.04 ± 0.28	0.82 ± 0.11
Smooth membranes	0.89 ± 0.15	1.92 ± 0.31	2.05 ± 0.14	1.58 ± 0.19

Distribution of enzyme activities in the fractions

Specific activities of some established marker enzymes were determined in the subcellular fractions from immature neurons (Table II). (Na^+–K^+)-activated ATPase, one of the best characterized markers of the plasma membrane[21], has a specific activity over 7-fold higher in the plasma membrane fraction than in the homogenate, while the smooth membrane fraction has an ATPase activity below the homogenate activity. Mitochondria have an (Na^+–K^+)-activated ATPase activity 1.7 times that of the homogenate and the other fractions only slightly above the level of the homogenate, with the exception of nuclei with low ATPase activity, 0.15 times that of the homogenate.

K^+-stimulated phosphatase and 5′-nucleotidase, which are preferentially localized in the plasma membranes of many tissues, are distributed in a rather similar manner between the subcellular fractions (Table II). Those two enzymes reach their highest specific activities in the plasma membrane fraction, over 3-fold that of the homogenate. These enzymes also show a high specific activity in the smooth membrane fraction, 2-fold the level of the homogenate, while the activity is again low in nuclei.

Acetylcholinesterase is thought to participate in humoral transmission, and fractionation of brain homogenate reveals that the activity of this enzyme is concentrated in the synaptosomal fraction but activity is found in other subcellular fractions as well (see ref. 22).

Table II shows the activities of cholinesterase in the subcellular fractions from immature neurons. The use of the specific inhibitor of acetylcholinesterase, 284C51, in the incubation medium decreased the specific activity of cholinesterase in all fractions to less than 20 % of the total activity. The values of the two enzymes in these fractions are therefore fairly close, most of the cholinesterase activity present being acetylcholinesterase. The highest specific activities are found in the synaptosomal fraction, but concentration is noted in the smooth membranes and processes as well. A low specific activity of cholinesterase is found in the nuclear fraction.

DISCUSSION

The subcellular fractionation of adult brain tissue is difficult compared to that of other tissues, because of its structural complexity. The techniques available yield enriched fractions of mitochondria, synaptosomes, myelin[1,2], smooth and rough endoplasmic reticulum[10,23] and axons[24]. Attempts to purify plasma membrane from brain have been limited to the isolation of axolemma from squid nerves[3,4], synaptosomal plasma membrane[5,6] and, recently to the preparation of neuronal plasma membrane from isolated neurons[7]. The difficulties in the latter approach using adult brain[7] are illustrated by the fact that these workers needed ten rabbits to obtain the same amount of rather contaminated plasma membrane preparation that we can isolate from one immature rat.

Owing to the technical difficulties in the isolation of plasma membranes, no specific markers for brain plasma membranes have established. On analogy to other tissues, it appears that electron microscopy, a high content of phospholipids, a low content of RNA, a concentration of (Na^+–K^+)-activated ATPase and, possibly, 5′-nucleotidase and K^+-stimulated phosphatase activity may be considered valid criteria for the identification of brain plasma membranes as well. Yet in the case of immature rat brain, in which enzyme activities are rapidly increasing[25], enzyme assays should be viewed with some caution, as the distribution of enzyme activities may be more heterogeneous than the fractions.

Electron microscopy of the plasma membrane preparation (Fig. 3) showed long membrane strands, while no myelin or synaptosomes were visible. The size of the membranes in the plasma membrane fraction was large compared to the small vesicles composing the smooth membrane fraction. The (Na^+–K^+)-activitated ATPase activity was concentrated over 7-fold and the 5′-nucleotidase and K^+-stimulated phosphatase over 3-fold as compared with the homogenate. The evaluation of purity of the plasma membrane fraction based on these data is not conclusive, as there is no indication that the above enzymes are exclusively localized in plasma membranes[26]. However, compared with other tissues, the concentration of ATPase activity in the plasma membrane fraction is of the same order of magnitude[21]. By contrast, the present data on electron microscopy and ATPase activity indicate that the plasma membranes are purer than in a similar isolation procedure from brain[7], and the total recovery is over 1000-fold higher.

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