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Comparison of Several Procedures for the Preparation of Synaptosomal Plasma Membrane Vesicles

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Abstract□Synaptosomal plasma membrane vesicles (SPMV) were isolated from fresh bovine cerebral cortex by several well-known procedures, and the best procedure was selected by enzymatic and morphological standards. The SPMV isolated by the modified procedure of Smith and Loh showed the highest purity. The specific activities of Na, K-ATPase, acetylcholinesterase and 5′-nucleotidase were about 5.6-fold, 2.5-fold and 3.3-fold, respetively, enriched in the plasma membrane fraction as compared to those of the crude homogenate. Electron microscopic examination also showed that the membranes were in vesicular form.

Keywords□Synaptosomal plasma membrane vesicles, sucrose density gradient, Ficoll density gradient, enzymatic and morphological evaluation.

Recently there have been increasing interests in the nature of drug-membrane interaction. For the study of the interaction, the purity of biomembranes should be crucial. Hence we are interested in the development of a new method for the isolation and purification of plasma membranes from intact cells. We isolated the synaptosomal plasma membrane vesicles (SPMV) by several well-known procedures with or without modifications and selected the best procedure on the basis of electron microscopic examination and enzymatic evaluation. The aim of this research is to develop an economical method for the isolation of high-purity plasma membranes in relatively good yield.

EXPERIMENTAL METHODS

Two-year-old Korean cattle of either sex were killed at Daeyoung slaughterhouse (#192 Kupodong, Buk-ku, Pusan, Korea). The whole brain was rapidly excised, placed in ice-cold SH (0.32 M sucrose + 3 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.5), and then immediately carried to our laboratory. All steps, subsequent to decapitation and removal of the cortex, were carried out at 0-4°C. All SPMV prepared by the following procedures were divided into small aliquots, quickly frozen in liquid nitrogen, and stored at −70°C.

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The procedures of Hajós and Kanner

Synaptosomes were isolated by Hajós[1], and plasma membrane vesicles were prepared by Kanner[2]. Bovine cerebral cortex (20g) was minced in 0.3 M sucrose, homogenized at 7-10% (w/v) with 10 strokes of a tissue grinder (Eberbach, USA), and then centrifuged in a Sorvall SS-34 rotor (Sorvall RC-5B centrifuge) at 1,500×g for 10 min. The pellet was discarded and the supernatant was recentrifuged at 9,000×g for 20 min. The supernatant was discarded and the pellet was dispersed in 5 m*l* 0.3 M sucrose, layered over 20 m*l* 0.8 M sucrose and centrifuged at 9,000×g for 25 min (plus a 5-min acceleration period). The particles dispersed in 0.8 M sucrose were saved, and the sucrose concentration was adjusted with water to 0.3 M sucrose. After centrifugation, 20 min

at 27,000×g, the pellet (synaptosomes) was lysed by resuspension in a minimal volume of 5 mM Tris-HCl + 1 mM K-EDTA (pH 7.4), followed by homogenization (3 strokes). After dilution in the same low osmolarity buffer, lysis was completed by stirring for 45 min. The lysate was centrifuged again, at 27,000×g for 20 min, and the pellet (SPMV) was resuspended in 0.32 M sucrose, 1 mM $MgSO_4$, 0.5 mM EDTA, 5 mM Tris-SO_4, pH 7.4 (buffer A) at a protein concentration of 5-10 mg/ml.

The procedure of Kanner

Bovine cerebral cortex (20g) was homogenized in ME (0.3 M mannitol + 1 mM K-EDTA, pH 7.4). After centrifugation of the homogenate at 3,000×g for 10 min, a crude mitochondrial pellet was obtained by centrifugation of the supernatant at 27,000×g for 15 min. The mitochondrial pellet was resuspended in ME and layered over discontinuous density gradients consisting of five layers of 20, 16, 12, 8 and 2% Ficoll in ME. The gradients were then centrifuged in a Beckman SW-28 rotor (Beckman L80M ultracentrifuge) at 58,000×g for 90 min. The 8-12% interfaces were collected and diluted with approximately 3 volumes of ME. After centrifugation at 27,000×g for 20 min, the synaptosomal fraction was obtained. The preparation of SPMV was identical to the procedures of Hajós[1] and Kanner[2].

A modification of the procedures of Hajós and Kanner

All steps were identical to the procedures of Hajós[1] and Kanner[2], except further purification by the sucrose density gradient centrifugation. The SPMV obtained by the procedures of Hajós[1] and Kanner[2] were resuspended in SE (0.32 M sucrose + 1 mM K-EDTA, pH 7.4) instead of buffer A, and layered over discontinuous density gradients comprising four layers of 1.3, 1.1, 0.9 and 0.7 M sucrose in 1 mM K-EDTA, pH 7.4. The gradients were centrifuged at 90,400×g for 45 min. Material sedimented to the 0.7 to 0.9 M and the 0.9 to 1.1 M sucrose interfaces was pooled, diluted with SE and centrifuged at 27,000×g for 20 min. The pellet (SPMV) was also resuspended in buffer A at a protein concentration of 5-10 mg/ml.

The procedure of modified Smith and Loh

Synaptosomes and plasma membrane vesicles were isolated by the method of Smith and Loh[3], modified as follows. Bovine cerebral cortex (20g) was placed in 150 ml of SH solution (pH 7.5). The cerebral cortex was homogenized with 10 strokes of a tissue grinder and then centrifuged at 1,200×g for 5 min. The pellet (P-1) was saved and the supernatant (S) was centrifuged at 17,300×g for 12 min. This second pellet (P-2) was washed once with SH and recentrifuged at 17,300×g for 12 min. The pellet (washed P-2) was resuspended in SH, layered over 7.5% (w/v) and 12% (w/v) Ficoll in SH, and centrifuged at 75,000×g for 60 min. Particles were resolved into the following fractions: (1) particles dispersed in 7.5% (w/v) Ficoll in SH, (2) particles at the 7.5% (w/v)-12% (w/v) interface, (3) particles dispersed in 12% (w/v) Ficoll in SH, and (4) the pellet (P-3). The fraction 2 was saved, diluted with SH, and centrifuged at 18,120×g for 20 min. The synaptosomal pellet was lysed by resuspension in 6 mM-Tricine (pH 8.1) and lysis was completed by stirring for 90 min. The suspension was layered over discontinuous density gradients consisting of 10, 20, 25 and 32.5% (w/v) sucrose containing 3 mM-Hepes, pH 7.5, and centrifuged at 16,400×g for 160 min. The SPMV-A (10-20%), SPMV-B (20-25%) and SPMV-C (25-32.5%) membrane bands were collected, diluted with water, and centrifuged at 30,620×g for 20 min. The pooled SPMV (SPMV-B + SPMV-C) were also resuspended in buffer A at a protein concentration of 5-10 mg/ml.

Enzyme assays

The Na, K-ATPase activity was determined by measuring the liberation of inorganic phosphate from ATP in 1 ml of medium containing 50 mM Tris-HCl, 100 mM NaCl, 10 mM KCl, and 3 mM $MgCl_2$ (pH 7.4) at 37°C. The membrane protein (about 100μg) was preincubated in the assay medium for 5 min, and the reaction was started with 3 mM-ATP. After 10 min of incubation, the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. Inorganic phosphate released was determined by the method of Fiske and SubbaRow[4]. The Na, K-ATPase activity was determined by subtracting the ATPase activity observed in the presence of 1 mM ouabain from the total activity. The specific activity of 5′-nucleotidase was determined in a 0.3 ml incubation mixture containing 50 mM glycine-HCl (pH 8.7), 0.2 mM EDTA, 6 mM AMP, 10 mM $MgCl_2$ and 15 to 60μg of membrane protein. The reaction was carried out at 37°C for 30 to 60 min. The incubation was terminated with an equal volume of 1% sodium dodecyl sulfate, and the liberated phosphate was determined with the Fiske-SubbaRow[4] reagent. Acetylcholinesterase was assayed as described by Ellman *et al.*[5], and the protein concentration was determined by the method of Lowry *et al.*[6] using bovine serum albumin as a standard.

Electron microscopy

Samples of SPMV, about 1 mg protein, were diluted with 0.3 M sucrose. One volume of 2% OsO_4 was added to three volumes of sample, and after 2 hr at 0-4°C, the fixed particles were sedimented in a bench centrifuge. Dehydration was carried out in the centrifuge tubes by treating the pellet consecutively with increasing concentrations of ethanol (50, 70, 80, 90 and 100%) at 0-4°C (10-15 min exposure to each solution). Dehydration was perfected with ethanol-acetone (1:1) and acetone alone (10-15 min each). The pellet was then removed from the tube and cut into small pieces which were transferred successively into mixtures of acetone and Epon (1:1 for 1 hr and 1:3 for 2 hr, respectively). The samples were embedded in Epon alone and kept overnight. Finally, Epon was renewed and kept at 60°C for 24 hr. Sections were cut with a Reichert ultramicrotome, stained with lead citrate-uranyl acetate, and examined with a Tesla 413 electron microscope. Unless otherwise specified, all steps were carried out at room temperature.

RESULTS AND DISCUSSION

The purity of SPMV was determined by enzymatic and morphological standards. Partial characterization of the SPMV fractions by enzyme assays (Table I) shows that the specific activities of Na, K-ATPase, acetylcholinesterase, and 5′-nucleotidase, were about 4 to 6-fold, 2.5-fold and 3-fold, respectively enriched in the plasma membrane fraction with respect to crude homogenates. Except for the procedures of Hajós[1] and Kanner[2], there was little difference in the enrichment of enzymatic activities among the procedures. This enrichment is quite small relative to the 20-25 fold purification of marker enzymes achieved for other tissue. Cotman and Matthews[7] explained that this is probably due to a higher proportion of plasma membranes in brain homogenates. Similar purification was obtained in SPMV isolated from rat brain[7] and from mouse brain[3].

It is generally accepted that Na, K-ATPase is a marker for the plasma membrane, but there are no reliable data as to its differential localization in glial and neuronal membranes. But it might be anticipated that the neuronal membrane, which has particular requirements for ion transport, would be enriched in the enzyme. 5′-Nucleotidase, a marker for the plasma membrane in some tissues[8], was enriched in these preparations, but to a lesser degree than was Na, K-ATPase. Histochemical evidence suggests that this enzyme is associated with certain types of glial plasma membranes[9]. This dual localization would explain

Table I. Comparison of enzymatic activities of synaptosomal plasma membrane vesicles isolated from bovine brain

SPMV	Na, K-ATPase	Acetylcholinesterase	5′-Nucleotidase
Homogenate	6.82 ± 0.59	5.62 ± 0.58	1.54 ± 0.16
SPMV[a]	28.86 ± 1.07	13.69 ± 0.85	4.37 ± 0.34
SPMV[b]	34.51 ± 1.45	14.14 ± 0.95	4.84 ± 0.44
SPMV[c]	36.02 ± 1.19	14.01 ± 0.97	4.97 ± 0.47
SPMV[d]	38.19 ± 1.29	14.21 ± 0.61	5.05 ± 0.31

Activities are expressed as μmoles of substrate utilized per milligram of protein per hour. Values represent the mean ± SEM of 5 determinations.

[a] The procedures of Hajós[1] and Kanner[2]

[b] The procedure of Kanner[2]

[c] A modififcation of the procedures of Hajós[1] and Kanner[2]

[d] The procedure of modified Smith and Loh[3]

the lower enrichment of this enzyme in these preparations. Acetylcholinesterase was even less enriched than the other marker enzymes. Although this enzyme has been used as a marker for the synaptic membrane, histochemical evidence suggests that the enzyme is also found in the endoplasmic reticulum, vesicular formations and neurotubules of many neurons, and that it is not present in all neuronal plasma membranes[10]. Thus, the low enrichment in these preparation is explicable, and the higher relative specific activities obtained in other studies are probably due to microsomal contamination.

In contrast with the results of enzyme assays, electron microscopic examination of the membranes showed significant differences. The SPMV prepared by Hajós[1] and Kanner[2] were of varying size, contaminated by intracellular organelles, and even showed leakage (Fig. 1). The advantage of this procedure is economical production of SPMV at low centrifugal forces. The procedure of Kanner[2] employs Ficoll density gradients and an ultracentrifuge. Although better than in Fig. 1, the electron micrograph showed vesicles of varying size and the presence of intracellular organelles (Fig. 2). In order to perform routine separations on a large scale and to prepare high-purity membranes, we developed a modification of the procedures of Hajós[1] and Kanner[2]. The membrane vesicles were further purified by discontinuous sucrose gradient. The selection of this gradient was based on the concentrations of sucrose required to separate plasma membrane from mitochondria and other membranes. Unfortunately, the vesicles were still of varying size and a few showed the presence of intracellular or-

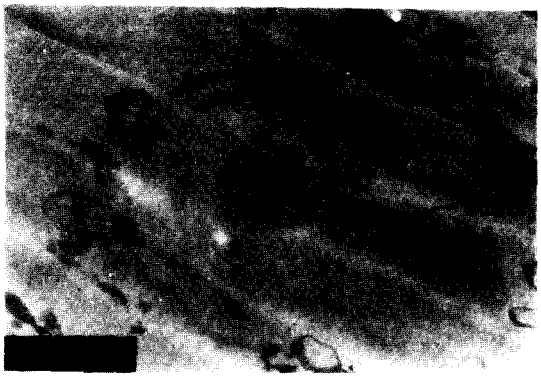

Fig. 1. Transmission electron micrograph (20,000 × 1) of synaptosomal plasma membrane vesicles isolated by the procedures of Hajós[1] and Kanner[2].

Fig. 2. Transmission electron micrograph (20,000 × 1) of synaptosomal plasma membrane vesicles isolated by the procedure of Kanner[2].

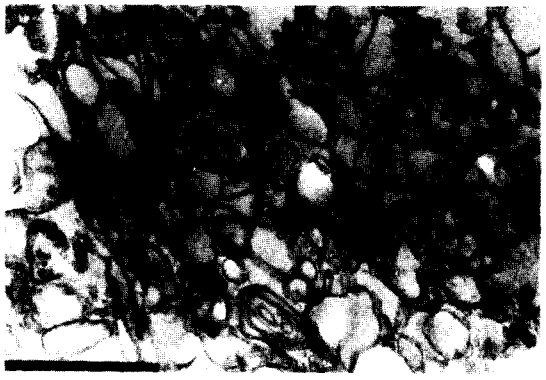

Fig. 3. Transmission electron micrograph (20,000 × 1) of synaptosomal plasma membrane vesicles isolated by a modification of the procedures of Hajós[1] and Kanner[2].

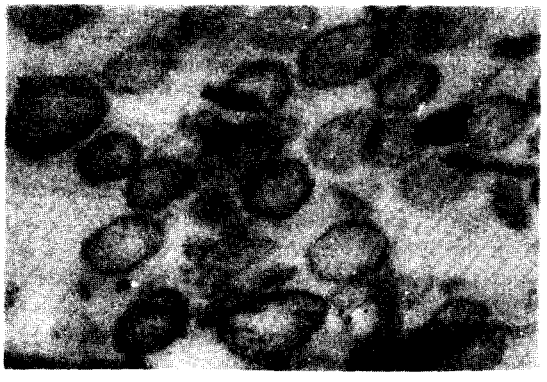

Fig. 4. Transmission electron micrograph (20,000 × 1) of synaptosomal plasma membrane vesicles isolated by the modified procedure of Smith and Loh[3].

ganelles (Fig. 3). However, it was notable that the purity of SPMV prepared by this procedure was higher than that obtained by Kanner[2]. Electron microscopic examination of the SPMV prepared by modified Smith and Loh[3] showed the highest purity. The vesicles were of a size, showed homogeneous distribution, and no longer showed the presence of intracellular organelles or leakage (Fig. 4). The black bar shown in Fig. 4 is merely due to contamination of lead compound, a staining agent. The enzymatic activities of synaptosomal subfractions are shown in Table II.

From these results, it is evident that synaptosomes are to be prepared by density gradient centrifugation on isotonic Ficoll-sucrose gradients and, after osmotic shock of the synaptosomes at alkaline pH, plasma membrane vesicles are to be isolated by a discontinuous sucrose density gradient centrifugation. In addition, electron microscopy afforded an excellent opportunity for judging the purity of the plasma membrane preparations.

Synaptosomes isolated on isotonic Ficoll-sucrose density gradients are highly sensitive to osmotic shock, whereas synaptosomes isolated by sucrose density gradients shrink under the hypertonic conditions and become less fragile. As pointed out by Cotman and Matthews[7], alkaline conditions during osmotic shock seem to favor a better separation of membrane from mitochondria, probably because of a more effective lysis of synaptosomes. The effect of alkaline buffers is not known, but may be a combination of factors, including an incubation effect. Related findings have been observed in other systems. The use of moderately alkaline conditions has been described as advantageous for the lysis of red blood cell[11], for the removal of loosely bound or adsorbed proteins from membrane[12], for promoting membrane vesicularization[13], and

Table II. Enzymatic activities of bovine brain subfactions prepared by the procedure of modified Smith and Loh

Subfraction	Na, K-ATPase	Acetylcholinesterase	5′-Nucleotidase
Homogenate	6.82 ± 0.59	5.62 ± 0.58	1.54 ± 0.16
P-1	7.03 ± 0.58	4.04 ± 0.47	1.30 ± 0.24
S	10.14 ± 0.67	5.76 ± 0.34	1.59 ± 0.14
P-2	13.50 ± 0.68	7.28 ± 0.75	1.69 ± 0.15
Washed P-2	15.28 ± 0.59	8.65 ± 0.95	1.83 ± 0.28
Fraction 1	17.29 ± 0.86	11.42 ± 0.94	1.95 ± 0.30
Fraction 2	15.46 ± 0.55	10.85 ± 0.40	1.85 ± 0.26
Fraction 3	13.14 ± 0.88	3.27 ± 0.53	1.24 ± 0.28
P-3	13.09 ± 0.76	3.09 ± 0.38	1.21 ± 0.16
SPMV-A	25.09 ± 1.56	11.60 ± 0.83	4.14 ± 0.39
SPMV-B	34.53 ± 0.70	14.06 ± 0.47	4.74 ± 0.32
SPMV-C	42.23 ± 1.69	14.45 ± 0.76	5.21 ± 0.30
Crude Synaptosomal mitochondria	4.77 ± 0.51	3.20 ± 0.33	1.09 ± 0.18
SPMV	38.19 ± 1.29	14.21 ± 0.61	5.05 ± 0.31

Activities are expressed as μ moles of substrate utilized per milligram of protein per hour. Values represent the mean ± SEM of 5 separate subfraction procedures.

for lysing zymogen granules[14].

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