SUBCELLULAR DISTRIBUTION IN CEREBRAL CORTEX OF TWO PROTEINS PHOSPHORYLATED BY A cAMP-DEPENDENT PROTEIN KINASE

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ABSTRACT

The subcellular distribution of Proteins Ia and Ib, two proteins which serve as specific substrates for protein kinases present in mammalian brain, was studied in the dog cerebral cortex. Proteins Ia and Ib were found to be most highly enriched in synaptic vesicle fractions; they were also present in postsynaptic density and synaptic membrane fractions in significant amounts. Proteins Ia and Ib present in the synaptic vesicle fraction appear to be similar, if not identical, to those present in the postsynaptic density fraction as judged by several criteria: (*a*) the ability to serve as substrate for cAMP-dependent protein kinase, (*b*) electrophoretic mobility in the presence of sodium dodecyl sulfate, (*c*) extractability with NH_4Cl or EGTA, and (*d*) fragmentation to electrophoretically similar peptides by a purified *Staphylococcus aureus* protease. In addition, the postsynaptic density fraction has been found to contain cAMP-dependent Protein Ia and Protein Ib kinase activity. The subcellular localization of Proteins Ia and Ib suggests a role for these proteins in the physiology of the synapse.

KEY WORDS cAMP · protein phosphorylation · proteins Ia and Ib · postsynaptic densities · synaptic vesicles · protein kinase

A variety of evidence indicates that adenosine 3′:5′-monophosphate plays an important role in mediating or modulating several types of neuronal function, including transmission at certain synapses (for reviews, see references 2, 7, 8, 11, 14, 15, 16, 20, 31). There is increasing evidence that many of the effects of cyclic AMP[1] in various tissues, including nervous tissue, are mediated through alteration of the state of phosphorylation of spe-

[1] *Abbreviations used in this paper are:* cAMP, adenosine 3′:5′-monophosphate; EGTA, ethyleneglycol-bis (β-amitoethyl ether)N,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; IBMX, isobutylmethylxanthine; MES, 2-(N-morpholino) ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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cific proteins (for reviews, see references 8, 14, 15, 20, 22, 25, 31, 33, 45. In subcellular fractions enriched in synaptic membranes, cAMP has a marked stimulatory effect on the phosphorylation of several endogenous substrate proteins, catalyzed by an endogenous protein kinase (10, 34, 36, 37). Two of these substrate proteins, Proteins Ia and Ib, collectively referred to as Protein I, appear to be specific to neuronal membranes (34). In addition to serving as a substrate for a cAMP-dependent protein kinase present in synaptic membranes, Protein I is also phosphorylated in intact synaptosomes in response to veratridine-induced or potassium-induced influx of Ca^{2+} ions (23). Protein I has recently been purified from calf cerebral cortex to apparent homogeneity and partially characterized (36). Purified Protein I from calf brain is a mixture of Protein Ia and Protein Ib in an approximate molar proportion of 1 to 2 (36). These two peptides are similar to one another and have unique properties; they are basic, elongated proteins that have a high content of glycine and proline, and are specific to nervous tissue. In the present paper the subcellular localization of Proteins Ia and Ib has been studied by biochemical methods.

MATERIALS AND METHODS

Subcellular Fractionation of Cerebral Cortex

The method for obtaining subcellular fractions from dog cerebral cortex was as given earlier (5); in addition, the method of Whittaker et al. (44) was used to obtain the synaptic vesicle fractions. A short description of how the fractions listed in Table I were obtained is noted in the flow sheet (Chart 1) and given below. Briefly, a homogenate was made in 0.32 M sucrose containing 1 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Primary subcellular fractions were obtained by differential centrifugation in the cold: a nuclear and cell debris pellet (1,400 g(Av) − 10 min); a crude mitochondrial plus synaptosomal pellet (13,000 g(aV) − 10 min); a microsomal pellet (105,000 g(Av) − 60 min); and a final supernate. The crude mitochondrial plus synaptosomal fraction was sedimented on a discontinuous sucrose density gradient (82,500 g(Av), 120 min in a Spinco SW 27 rotor [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.]), to give an upper floating myelin fraction, and two bands, one a mixture of membranes and the other the synaptosomal fraction, and finally a pelleted mitochondrial fraction. The synaptosomal fraction was lysed by osmotic shock, and the lysate was spun at 32,800 g(Av) − 20 min in a Sorvall SS-34 rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) to give a crude synaptic membrane fraction pellet. The supernate from this lysate was then spun at 78,000 g(Av) − 120 min in a Beckman-Spinco 30 rotor, to give a crude synaptic vesicle fraction pellet. The latter was resuspended and centrifuged (64,700 g(Av), 120 min, in a Spinco SW 25.1 rotor) on a discontinuous sucrose density gradient (cf. reference 46) to give four synaptic vesicle fractions, of various degrees of purity (cf. Figs. 3 *a–d*). Synaptic vesicle fraction 1 contained the material in 0.4 M sucrose plus the band at the 0.4–0.6 M sucrose interface; synaptic vesicle fraction 2 contained the material in 0.6 M sucrose plus the band at the 0.6–0.8 M sucrose interface; synaptic vesicle fraction 3 contained the material in 0.8 M sucrose; and synaptic vesicle fraction 4 was the pellet. The crude synaptic membrane fraction was also purified by discontinuous sucrose-density gradient centrifugation (82,500 g(Av), 120 min in a Spinco SW 27 rotor) to give three synaptic membrane fraction bands of various degrees of purity, plus a pellet of intrasynaptosomal mitochondria. The lower synaptic membrane fraction, at the 1.0–1.2 M sucrose interface, of higher purity than the others (cf. Fig. 3 *f*), was then pelleted, resuspended, and treated with 0.5% Triton X-100 for 15 min. The Triton lysate was then centrifuged to give a mixed postsynaptic density (PSD) and membrane pellet plus a supernate. The pellet was suspended and centrifuged on a sucrose density gradient (201,800 g(Av), 120 min in a Spinco SW 40 rotor) to give three bands, an upper membrane fraction, a lower purified PSD fraction (cf. Fig. 3 *e*), and a middle band containing a mixture of the materials in the other two bands.

Assay of Protein I

Various subcellular fractions (2–4 mg protein/ml) were treated with 0.1 M NH_4Cl at 4°C for 30 min; this procedure has been shown (36) to extract Protein I from a synaptic membrane preparation. The extracted proteins were obtained by centrifugation at 150,000 g for 30 min. The pellet was suspended in water and subjected to re-extraction with 0.1 M NH_4Cl. Those subcellular fractions that had not been subjected to osmotic shock were homogenized in 9 vol of cold water and centrifuged at 150,000 g for 30 min before extraction; in such cases, the pellet was suspended in water and then subjected to NH_4Cl extraction, as described above. As noted before with a synaptic membrane preparation (36), two extractions were found sufficient to extract most (80–90%) of Protein I from the various fractions. The two extracts were combined and assayed for Protein I, as described previously (36); Protein I was assayed for its ability to serve as a substrate for cAMP-dependent protein kinase. Briefly, 10–30 μl of the NH_4Cl extract were incubated at 30°C for 30 min in a final volume of 100 μl in the presence of 1 μM [γ-^{32}P]ATP (7.5–20 Ci/mmol), 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 μM cAMP, 1 mM IBMX, 10 mM NaF, and 8 units of protein kinase prepared from bovine brain through Step 3 of the procedure of Uno et al. (38). This procedure assured maximal phosphorylation of Protein I; doubling the incubation time or increasing the protein kinase concentration twofold gave a virtually identical extent of phosphorylation of Proteins Ia and Ib. Since this protein kinase preparation did not show detectable Protein I phosphatase activity under the above incubation conditions, we assumed that this protein kinase preparation was adequate for the present study. The phosphorylation reaction was terminated by the addition of 50 μl of "SDS-stop solution" containing 9% SDS, 6% mercaptoethanol, 15% glycerol, and a small amount of bromphenol blue dye in 0.186 M Tris-HCl (pH 6.7). The phosphorylated proteins were subjected to SDS-PAGE on 8% polyacrylamide gels, according to the procedure of Laemmli (24), and autoradiography. The amount of [^{32}P]phosphate incorporated into Proteins Ia and Ib was quantitated as described previously (36). Autoradiographs were scanned with a Canalco G-II microdensitometer. The relative peak heights of the optical density tracings in the positions corresponding to Protein Ia and Protein Ib were used to estimate the relative amounts of [^{32}P]phosphate incorporated into Proteins Ia and Ib. A band containing both

Proteins Ia and Ib was cut from one slot of each of the dried slab gels, dissolved in Protosol, and radioactivity was determined in a liquid scintillation spectrophotometer, thus providing a means for the conversion of the arbitrary units obtained by the densitometric measurements to conventional radioactivity measurements.

Although the extraction procedure involving the use of acid, as described in a previous study (36), had the advantage over the NH_4Cl extraction procedure of inactivating endogenous protein kinase and protein phosphatase, the acid extraction procedure was not feasible in a situation in which very limited quantities of samples (e.g., highly purified postsynaptic density and synaptic vesicle fractions) were available for the assay of Protein I. Therefore, the NH_4Cl extraction procedure was adopted in the present study; this procedure gave results, in a preliminary study with some subcellular fractions, similar to those obtained with the acid extraction procedure. This suggests that the state of phosphorylation of Proteins Ia and Ib in the NH_4Cl extract before phosphorylation with [γ-^{32}P]ATP is the same as or similar to that of the Proteins Ia and Ib in the acid extract. The acid extraction procedure has been noted (27) to give Protein I entirely in the dephosphorylated form, so that the quantitation method for total Protein I, as used previously (36) and here, seems to be reliable. However, the possibility that the assay in which the NH_4Cl extraction procedure is used may have been interfered with by protein phosphatase or by protein kinase inhibitor, which might have been present in some subcellular fractions, cannot be excluded.

Phosphorylation of Proteins in Subcellular Fractions

The various fractions, each containing 60 μg of protein, were incubated for 3 min at 30°C in 30 mM MES buffer, pH 6.2, containing 10 mM Mg^{2+}, 1 mM IBMX, 8 units of cAMP-dependent protein kinase from bovine brain prepared through Step 3 of Uno et al. (38), 1–2.5 μM [γ-^{32}P]ATP (5–20 Ci/mmol), with or without 10 μM cAMP, in a final volume of 100 μl. (The assay mixture contained exogenously added purified protein kinase and cAMP, so as to allow only Protein I to be the limiting factor.) The reaction was stopped by the addition of 50 μl of "SDS-stop solution." It was found that the phosphorylation of Proteins Ia and Ib in the PSD reached a maximal value at between 1 and 2 min of incubation and remained at this level for at least 5 min of incubation.

In those experiments dealing with the extractability of Proteins Ia and Ib from the subcellular fractions, the incubation mixture—after the 3 min of incubation—was diluted ten times with either 0.32 M sucrose/1 mM $NaHCO_3$, pH 8.1 (sol. B), or 100 mM EGTA in sol. B, and adjusted to pH 8.1. The suspension was then kept for 2 h in the cold to achieve a complete extraction of Protein I by EGTA. The 2-h incubation did not seem to result in significant dephosphorylation of Proteins Ia and Ib as judged by visual comparison of autoradiographs of nontreated and treated PSD fractions. After centrifugation at 160,000 *g* for 30 min, the residue was saved and the supernate was made 10% (wt/vol) with regard to trichloroacetic acid (TCA) and kept for 2 h on ice. The TCA-precipitated proteins were centrifuged in an Eppendorf microfuge for 30 min and the pellets were then washed once with cold 5% (wt/vol) TCA and once with cold water. The pellets obtained after the 160,000 *g* centrifugation and the TCA-precipitated supernatant samples, as well as the nontreated subcellular fractions, were prepared for and run in a SDS-PAGE system different from the one described above, using a discontinuous buffer system and a polyacrylamide gradient as described earlier (5). The gels were stained for protein with Coomassie blue and then dried and subjected to autoradiography (Cronex 2,[h] Medical X-ray Film) for 15 h.

Electron Microscopy

Pellets of the synaptic membrane, postsynaptic density and synaptic vesicle fractions were initially fixed by immersion in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) and then in 1% osmium tetroxide in 0.03 M barbital buffer (pH 7.4). They were then stained en bloc in 4% uranyl acetate in 0.03 M barbital buffer (pH 7.4), dehydrated by standard procedures, and embedded in Epon which was polymerized at 60°C for 3 d. Thin sections were cut on a Porter-Blum microtome MT2B (DuPont), and then stained sequentially with 8% uranyl acetate and 4% lead citrate. All sections were examined on the HU-11B Hitatchi electron microscope.

RESULTS

Phosphorylation of Proteins Ia and Ib in Subcellular Fractions

Fig. 1 shows the results of an experiment on phosphorylation of Proteins Ia and Ib in two subcellular fractions (cf. Chart I), the purified PSD fraction (cf. Fig. 3 *e*) and the crude synaptic vesicle fraction. The protein staining profiles of the two fractions (Fig. 1) were quite different, and these differences were quite reproducible. After incubation with radioactive ATP, many of the proteins of these two fractions were phosphorylated (Fig. 1); indeed, when the gels were exposed for a longer period of time, it was observed that most of the protein bands were phosphorylated. However, the phosphorylation of only two proteins (Proteins Ia and Ib; see below) was greatly increased by the addition of cAMP, those whose M_r was estimated to be 73,000 and 68,000 in the continuous gradient gel system used in this experiment (cf. Materials and Methods). Lanes 5 and 6 show this for the two proteins in the PSD fraction, and Lanes 7 and 8 show this for the crude synaptic vesicle fraction. This type of experiment was repeated many times with similar results. Occasionally, the phosphorylation of two other proteins (26,000 and 60,000 mol wt) was also increased by the addition of cAMP.

In addition to serving as prominent substrates for cAMP-dependent protein kinase, Proteins Ia and Ib in the PSD fraction have several other properties suggesting that they are similar to Proteins Ia and Ib in the synaptic vesicle fraction. First, the 73,000 and 68,000 mol wt proteins in the PSD and the synaptic vesicle fractions gave identical gel mobilities (Fig. 1), and also moved the same way in the same gel system as purified

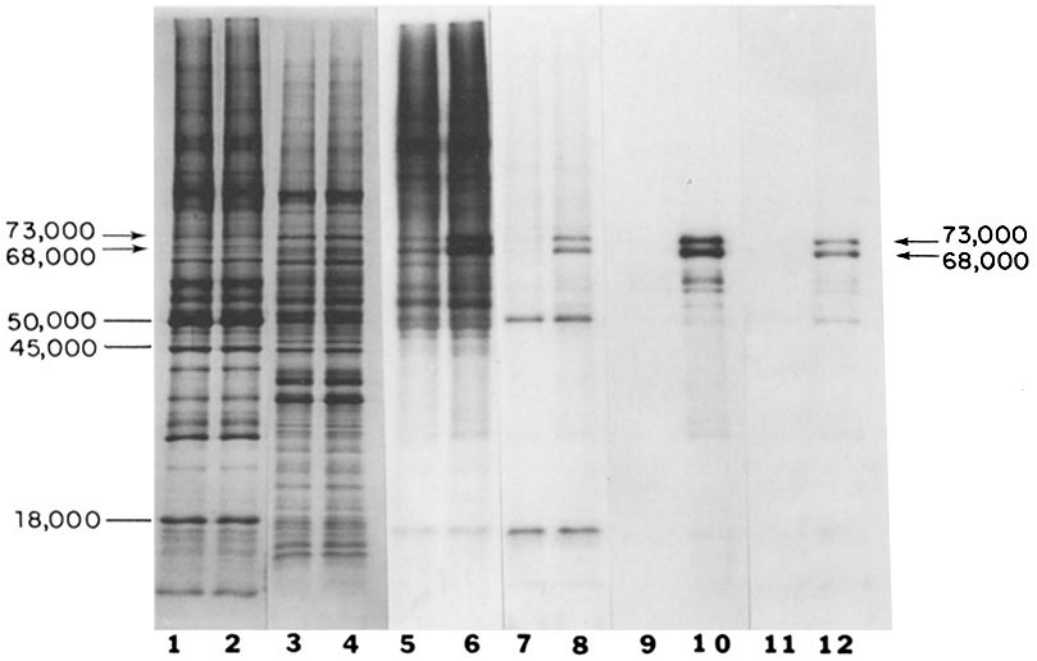

FIGURE 1 Phosphorylation of proteins in the postsynaptic density and crude synaptic vesicle fractions. The fractions were isolated as shown in Chart I and described in Materials and Methods, and were incubated as described in Materials and Methods, with partially purified protein kinase added to all the fractions and cAMP added as indicated below. The samples were treated with SDS and electrophoresed as described in Materials and Methods under *Phosphorylation of Proteins in Subcellular Fractions.* The Coomassie Blue-stained protein profiles are shown in slots 1 and 2 for 60 μg of the postsynaptic density fraction and in slots 3 and 4 for 60 μg of the crude synaptic vesicle fraction. Slots 5 and 6 show the autoradiograms of the same postsynaptic density fraction and slots 7 and 8 show the autoradiograms of the same crude synaptic vesicle fraction. The gels shown in slots 1, 3, 5, and 7 are of fractions incubated in the absence of cAMP, while slots 2, 4, 6 and 8 are of fractions incubated in the presence of 10 μM cAMP. Replicate samples of the postsynaptic density and crude synaptic vesicle fractions were incubated in the presence of both protein kinase and cAMP, and were then treated with either Solution B or 100 mM EGTA in Solution B as given in Materials and Methods. The treated samples were centrifuged and the pellets and supernates were prepared for gel electrophoresis as described in Materials and Methods. Slot 9 shows the autoradiogram of the postsynaptic density supernate after treatment with Solution B, and slot 10 shows it after treatment with EGTA in Solution B; slot 11 shows the autoradiogram of the crude synaptic vesicle supernate after treatment with Solution B, and slot 12 shows it after treatment with EGTA in Solution B. Not shown in the figure are the pelleted residues after the extraction treatments. These indicated that Proteins Ia and Ib were still in the postsynaptic density and vesicle residues after Solution B treatment but were absent from these residues after treatment with EGTA in Solution B. The arrows indicate the positions of Proteins Ia and Ib. The apparent molecular weight markers are derived from previous publications (3, 5).

Proteins Ia and Ib (not shown), prepared as described earlier (36). (These proteins had apparent molecular weights of 86,000 and 80,000 in the Laemmli gel system used earlier [36]; because density gradient centrifugation analysis gave a molecular weight of 37,000 [for a globular protein of 2.9 S], it was suggested that the protein has an elongated shape and thus would show different electrophoresis in different gel systems. Feit et al. [12] came to the same conclusion regarding the estimation of molecular weight values of neurofilament protein.)

Second, Proteins Ia and Ib from the PSD and synaptic vesicle fractions showed similar extractability by EGTA. Thus, the gels shown in Fig. 1 illustrate the results of an experiment in which the

CHART I

SUBCELLULAR FRACTIONATION OF CEREBRAL CORTEX

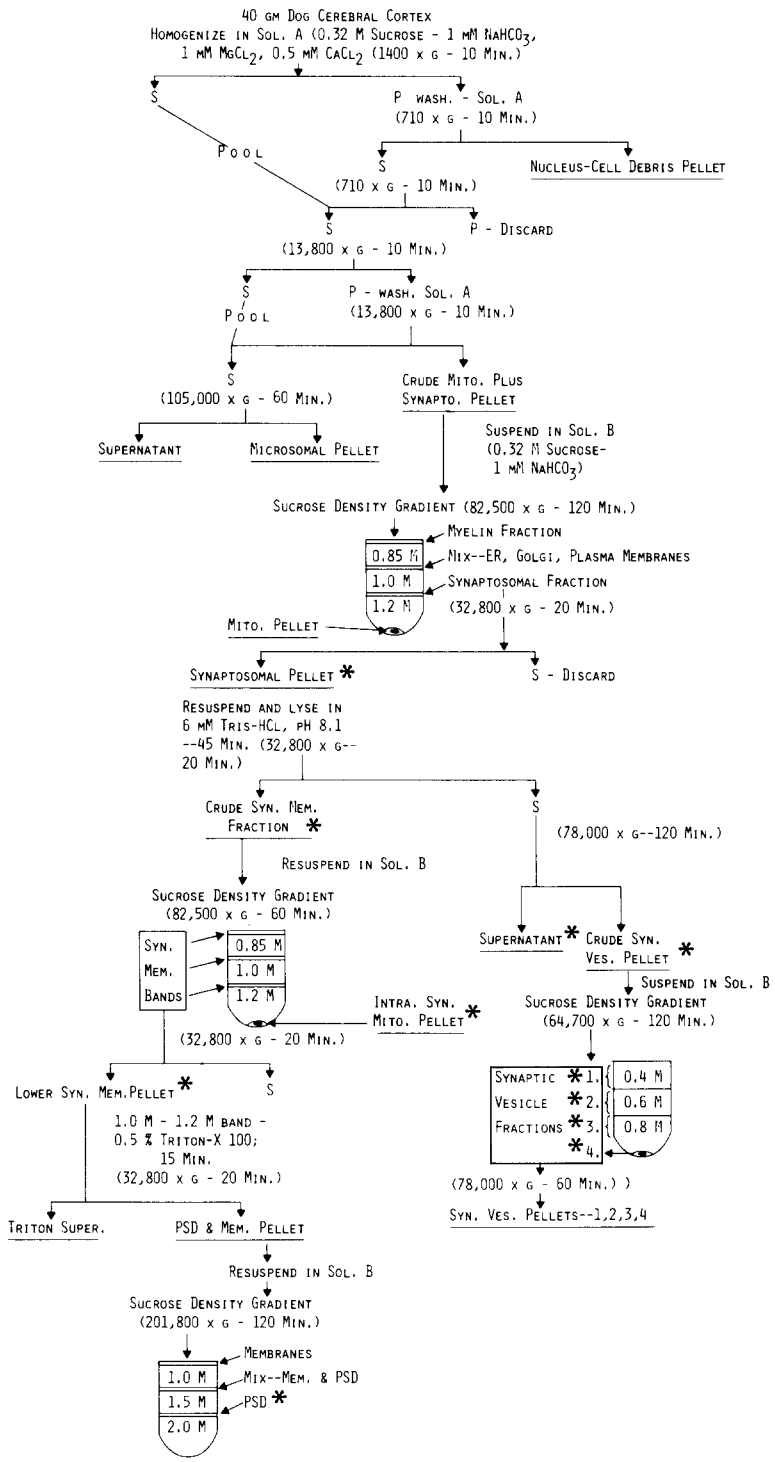

CHART I Fractionation Scheme. The g values given are average values. Fractions denoted by asterisks are those tabulated in Table I.

phosphorylated PSD and synaptic vesicle fractions were incubated in either sucrose/$NaHCO_3$, pH 8.1, alone, or sucrose/$NaHCO_3$ supplemented with 100 mM EGTA, pH 8.1, and then sedimented; the resulting supernates and pellets were analyzed by SDS gel electrophoresis. As shown in the autoradiograph of Fig. 1, EGTA extracted the phosphorylated 73,000 and 68,000 mol wt proteins from the PSD fraction (Slot 10) and also from the synaptic vesicle fraction (Slot 12). Treatment with a solution containing 0.32 M sucrose and 1 mM $NaHCO_3$, pH 8.1, extracted these two proteins only in small amounts from the PSD fraction (Slot 9) and from the crude synaptic vesicle fraction (Slot 11). Previously (3) it has been noted, using SDS-PAGE, that EGTA treatment of a PSD preparation released all of two minor Coomassie-blue-stained 73,000 and 68,000 mol wt proteins.

Third, when Protein I (Protein Ia plus Protein Ib) was extracted by NH_4Cl from the PSD and synaptic vesicle fractions and then was phosphorylated and subjected to limited proteolysis by *Staphylococcus aureus* protease in the presence of 0.1% SDS, similar if not identical digestion patterns were obtained (Fig. 2). Thus, the similarities in electrophoretic mobility, extractability by EGTA and by NH_4Cl, and protease digestion products support the notion that Proteins Ia and Ib in the postsynaptic density are the same as those in the synaptic vesicles.

The autoradiograph of Fig. 1, and similar results obtained in other experiments, would indicate that the amounts of Proteins Ia and Ib are higher in the PSD than in the crude synaptic vesicle fraction. However, it should be emphasized that the results of this type of experiment are only qualitative in character and only show the existence of the same proteins in these two fractions. Some reasons for this are as follows: differences in ATPase, in protein kinase, or in protein phosphatase activity between the two fractions, or accessibility of Proteins Ia and Ib to protein kinase or protein phosphatase, may exist and could markedly affect the amount of radioactive phosphate incorporated into these proteins in the intact organelles. For these reasons, another type of experiment was performed. This involved quantitatively extracting Proteins Ia and Ib from each of the subcellular fractions, phosphorylating them, subjecting the extracts to SDS-PAGE, and quantitating the amounts of Proteins Ia and Ib as described previously (36).

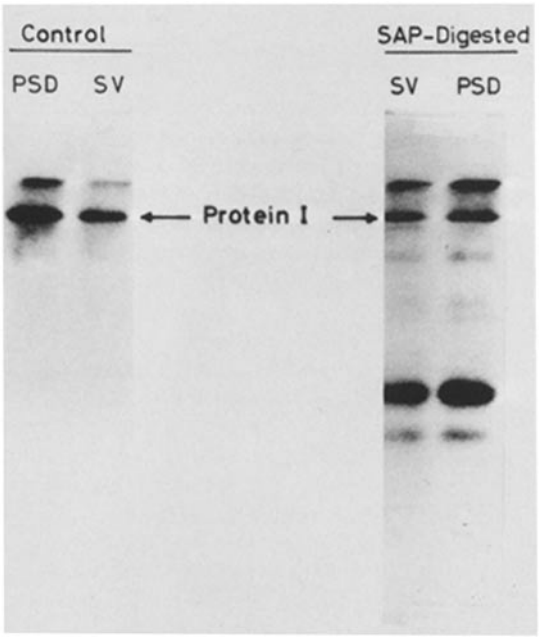

FIGURE 2 Limited proteolysis by *Staphylococcus aureus* protease of Protein I in the postsynaptic density and synaptic vesicle fractions. NH_4Cl extracts (cf. reference 36) obtained from the postsynaptic density (PSD, 45 μg for control and 90 μg for proteolytic digestion) and crude synaptic vesicle (SV, 30 μg for control and 60 μg for proteolytic digestion) fractions were phosphorylated and subjected to SDS-gel electrophoresis as described in Materials and Methods under *Assay of Protein I*. The gel was stained, destained, and dried, and subjected to autoradiography to identify the positions of Protein Ia and Protein Ib in the gel. The portion of the gel containing both bands was cut out, immersed in 0.125 M Tris-HCl (pH 6.8) for 30 min with occasional shaking, and subjected to a second SDS-gel electrophoresis (4) at 60 V for 15 h in the presence or absence of Miles Laboratory *Staphylococcus aureus* V8 protease (SAP) (0.1 μg/slot). The gel was dried and subjected to autoradiography. In this second SDS-gel system, the stacking gel (3.6%) and separating gel (15%) were 5 and 7 cm long, respectively. This electrophoretic system, which was used to permit separation of lower molecular weight peptides, did not separate Proteins Ia and Ib from each other. The phosphorylated protein band seen above Protein I is probably an aggregated form of Protein I stacked between the end of the stacking gel and the beginning of the separating gel.

Assay of Proteins Ia and Ib in Extracts of Various Subcellular Fractions

The results of subcellular fractionation of cere-

bral cortex, performed as described in Materials and Methods and in Chart I, and utilizing the quantitative extraction approach, are shown in Table I, while Fig. 3 shows electron micrographs of some of the fractions obtained thereby. Table I gives some of the data from an experiment in which all of the subcellular fractions were assayed for the concentrations of Proteins Ia and Ib. Not shown in Table I are the data from the first two steps of the fractionation involving crude heterogeneous fractions (cf. Chart I); in the first step, three pelleted fractions (nuclear and cell debris; crude mitochondria and synaptosomes; microsomes) and a supernate were obtained; in the second step, discontinuous sucrose density gradient centrifugation of the crude mitochondrial-synaptosomal fraction gave three bands (myelin; a mixture of endoplasmic reticulum, Golgi complex, and plasma membranes; synaptosomes) and a mitochondrial pellet. Also not shown are the data obtained with the various synaptic membrane bands and with the bands in the final postsynaptic density isolation (cf. Chart I). All these fractions had much lower concentrations of Protein I than did the synaptic vesicle, synaptic membrane, and PSD fractions listed in Table I. In the first step, the additive recovery from whole homogenate was 100% protein, and 65 and 90% for Proteins Ia and Ib; in the second step, the additive recovery from the crude mitochondrial and synaptosomal fraction was 45% for protein and 75% for both Proteins Ia and Ib. In other experiments, not all the fractions were assayed, but the data gave the same relative quantitative results as those shown in Table I, namely, that of the subcellular fractions, the synaptic vesicle fractions had the highest concentrations of Proteins Ia and Ib, followed in declining order by the postsynaptic density fraction and by the synaptic membrane fraction. Even the crude synaptic vesicle fraction had a high specific content of these two proteins (Table I). When this fraction was further fractionated on a sucrose density gradient, the lightest resultant band (synaptic vesicle fraction 1) had by far the highest concentrations of Proteins Ia and Ib, with the heavier bands declining in their specific content. Fig. 3 *a–d* show electron micrographs of the sedimented pellets of these four synaptic vesicle fractions. It is clear that vesicle fraction 1, having the highest concentrations of Proteins Ia and Ib, is also the purest band with regard to synaptic vesicles. Indeed, the decrease in specific content of the two proteins in the four fractions mirrors qualitatively the decrease in the proportions of synaptic vesicles in these fractions.

TABLE I

Specific Amounts of Proteins Ia and Ib in Various Subcellular Fractions from Dog Cerebral Cortex

Fraction	Protein Ia	Protein Ib
	pmol/mg protein	
Homogenate	6.2	5.1
Synaptosomal	9.6	13.7
Synaptosomal supernate	not detected	not detected
Crude synaptic membrane (after lysis)	6.6	9.9
Intrasynaptosomal mitochondria	5.4	8.1
Lower synaptic membrane (with PSD)	16.1	23.4
Postsynaptic density	23.2	35.6
Crude synaptic vesicle	27.8	39.1
Synaptic vesicle fraction 1	98.8	162.1
Synaptic vesicle fraction 2	46.0	83.5
Synaptic vesicle fraction 3	19.9	27.6
Synaptic vesicle fraction 4	14.0	19.2

The fractions listed here are marked by asterisks in Chart I, where is described the methodology used in obtaining them.

The purity of the postsynaptic density fraction, that fraction having the next highest concentration of Protein I, is attested to by the high proportion of intact postsynaptic densities seen in Fig. 3 *e*, and the relative lack of myelin, mitochondria, and synaptic vesicle contamination, in agreement with earlier observations (5). The indiscrete material seen in Fig. 3 *e* is thought to be either broken PSDs and PSDs sectioned tangentially, or the subsynaptic material adhering to the central PSD mass, as has been explained earlier (5).

While it is clear that the synaptic vesicle and PSD fractions are highest in their concentrations of Proteins Ia and Ib, the situation with regard to the synaptic membrane fraction is less clear. Fig. 3 *f* shows an electron micrograph of the lower synaptic membrane band obtained by density gradient centrifugation of a crude synaptic membrane fraction obtained from a lysate of synaptosomes (cf. Chart I). While there is contamination by some mitochondria and some synaptic vesicles, the major membrane component is probably synaptosomal plasma membranes (cf. references 17, 19, 26, 30, 39). This fraction also had a high specific content of Proteins Ia and Ib, two to five times higher than that of the original homogenate, and this value does not appear to be due to the small

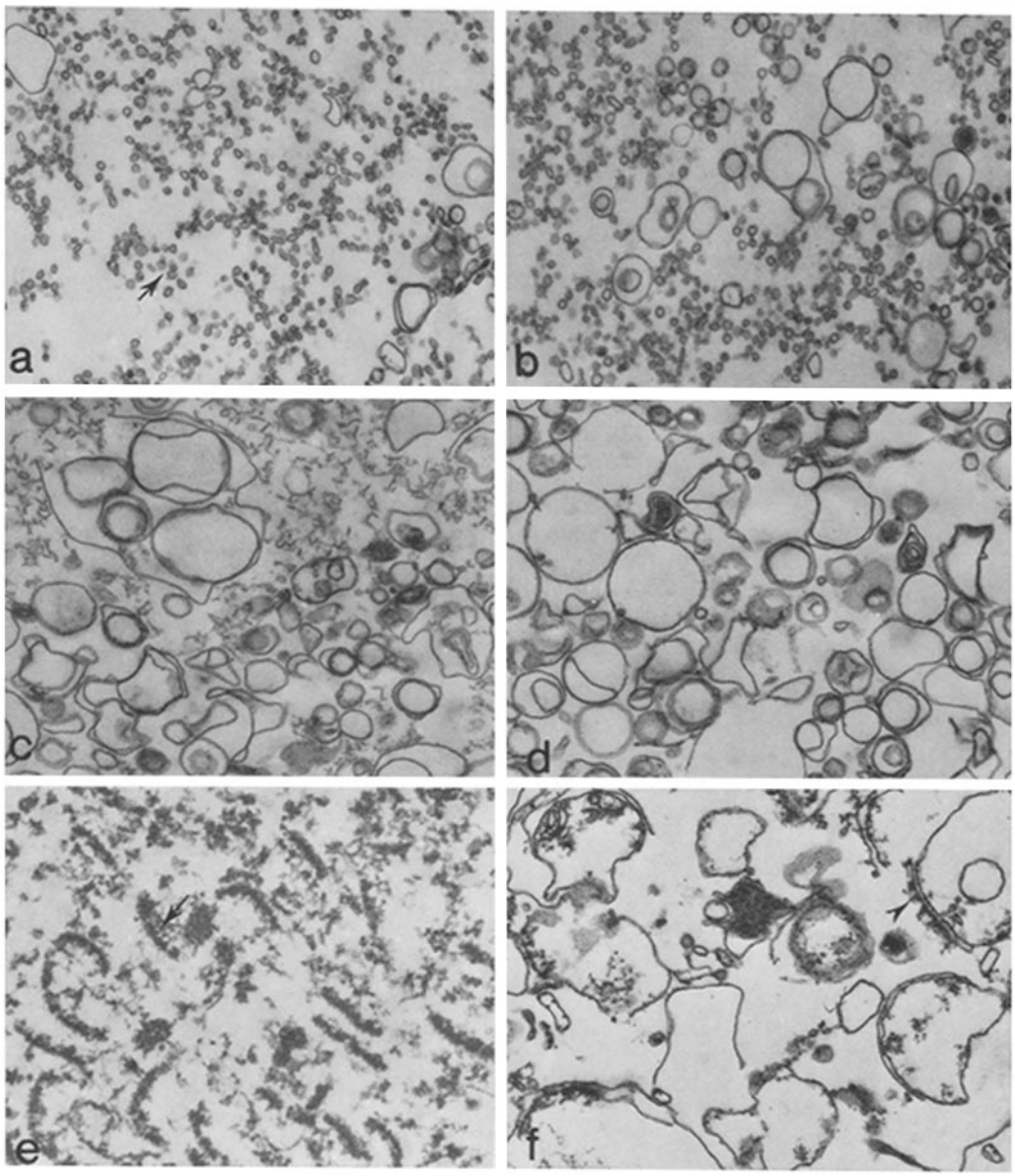

FIGURE 3 Electron micrographs of various subcellular fractions. (*a–d*) Various synaptic vesicle fractions taken from the gradient (cf. Chart I). × 25,000. (*a*) The 0.4 M sucrose band contains predominantly synaptic vesicles of 400–500 Å Diam (arrow) with occasional larger vesicular membranes of varying dimensions. This layer corresponds to the fraction (synaptic vesicle fraction 1) with the highest specific amount of Proteins Ia and Ib. (*b*) The 0.6 M sucrose band, in which the number of large vesicular membranes increases. (*c*) The 0.8 M sucrose band; synaptic vesicles are rarely seen, but the band contains many large membrane vesicles and some unidentified membranous fragments. (*d*) The pellet, which is almost completely devoid of synaptic vesicles. Large membrane vesicles predominate. (*e*) Postsynaptic density fraction. Single arrow indicates PSDs with attached subsynaptic web material. This fraction is high in its specific content of Proteins Ia and Ib. × 25,000. (*f*) Synaptic membrane fraction (Lower Synaptic Mem. fraction of Chart I). This fraction contains synaptosomal plasma membranes, some with recognizable synapses, as indicated by the arrow. This fraction has a high specific content of Proteins Ia and Ib, although not so high as that of the synaptic vesicle and PSD fractions. × 20,000.

amount of contamination with synaptic vesicles nor the small amount of PSDs still adhering to the postsynaptic membrane; it has been estimated (5) that the amount of PSDs in this fraction is ~5%, and electron micrographs (Fig. 3*f*) show only a very small number of synaptic vesicles in this synaptic membrane fraction. At present, we conclude that Proteins Ia and Ib are present in the synaptic plasma membrane fraction in higher concentrations than in the original homogenate, but not so high as in the synaptic vesicle and PSD fractions; we cannot exclude the possibility that the contents of Proteins Ia and Ib in this fraction are due to contamination by synaptic vesicles and/or PSDs.

Presence of Cyclic AMP-Dependent Protein I Kinase in Postsynaptic Density Fractions

In the phosphorylation experiments described above, an exogenous protein kinase was added to the reaction mixtures. If the exogenous protein kinase was omitted, Proteins Ia and Ib in the PSDs could still be phosphorylated in a cAMP-dependent manner (Fig. 4, compare lanes 1 and 2). The addition of cAMP alone to the PSD preparation was sufficient to elicit optimal phosphorylation of Proteins Ia and Ib; the addition of exogenous purified protein kinase did not elicit a further increase in phosphorylation (Fig. 4, compare lanes 2 and 3). These results indicate that the PSD has an intrinsic cAMP-dependent protein kinase. Further evidence that the PSD contains cAMP-dependent protein kinase was obtained in experiments with 8-N_3-[^{32}P]cAMP, a photo-affinity analogue which quantitatively labels the Type I and Type II cAMP-dependent protein kinases, both in cytosol and in membrane fractions of mammalian tissue (40, 41). With the use of this procedure, purified PSD fractions were found to incorporate 0.22 pmol of 8-N_3-[^{32}P]cAMP into the Type I cAMP-dependent protein kinase and 0.37 pmol into the Type II cyclic AMP-dependent protein kinase per mg of PSD protein (U. Walter and P. Greengard, unpublished experiments). Of relevance to these observations, it has previously been found (21, 42) that cyclic AMP-dependent protein kinase activity is present in a Triton X-100 insoluble synaptic junction complex.

When similar phosphorylation experiments were performed with the crude synaptic vesicle preparation, the addition of cAMP alone also elicited phosphorylation of Proteins Ia and Ib. However, when purified synaptic vesicle preparations were used, the results were variable; in about one-half the cases, the addition of cAMP alone increased the phosphorylation of Proteins Ia and

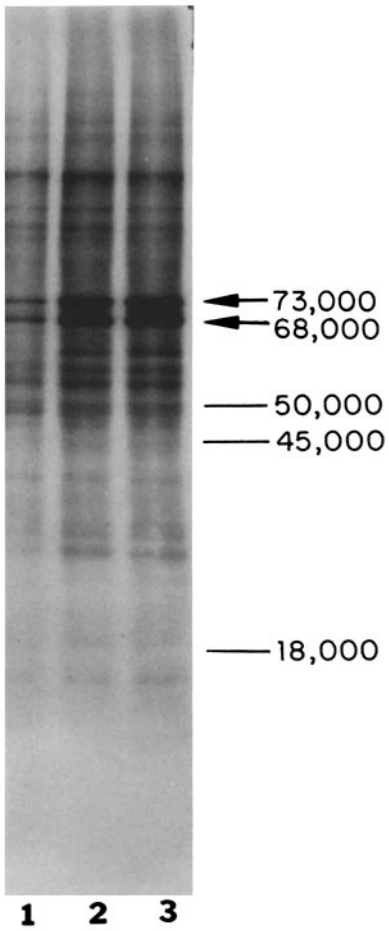

FIGURE 4 Autoradiogram indicating presence of a cAMP-dependent protein kinase in postsynaptic densities. The composition of the reaction mixture, containing 60 μg of PSD protein, is given under Materials and Methods, but with the addition of cyclic AMP and protein kinase only where noted. The reactions were stopped by the addition of TCA to a final concentration of 30% (wt/vol), and the precipitated proteins were subjected to SDS-gel electrophoresis and autoradiography, as described in Materials and Methods under *Phosphorylation of Proteins in Subcellular Fractions.* PSDs were incubated as follows: Lane 1, without cAMP or protein kinase; Lane 2, with 10 μM cAMP, but without protein kinase; Lane 3, with 10 μM cAMP plus 8 units of protein kinase. The arrows indicate the positions of Proteins Ia and Ib. The apparent molecular weight markers are derived from previous publications (3, 5).

Ib, while at other times no effect was observed, and no reason for the variation can be given at present. Thus, at this time we cannot make any definitive statement regarding the association of cAMP-dependent protein kinase with synaptic vesicles.

DISCUSSION

The isolation of purified fractions from brain, in good yield, is not yet at the stage of development of that for several other tissues such as liver. The problems encountered in brain tissue are the heterogeneity of cell types and the difficulty of isolating pure membrane fragments whose cellular origin is known (cf. review by Jones [18]). Nevertheless, it is possible to obtain some membrane fractions containing predominantly the subcellular elements in question, as judged by enzymatic marker studies, and thus, by means of relative specific activities, or contents, to establish whether the component, enzyme or protein in question, is in one or another of the subcellular organelles. Of all the fractions of brain, the synaptic vesicle fraction (29) and the postsynaptic density fraction (5, 6) are probably the most pure. They appear to be >90% pure, as judged mostly by electron microscopy and by relative lack of contaminant marker membrane enzymes (5). Our electron micrograph of the most purified synaptic vesicle fraction (Fig. 3 *a*) shows that most of the material is indeed synaptic vesicles with contamination by only a small amount of much larger membrane vesicles of unknown origin.

The electron micrograph of the PSD fraction (Fig. 3 *e*) again gives some idea of the high purity of this fraction. In addition, previous work (5) has shown that, by phospholipid analysis, at most only 2% of the protein in the PSD fraction is due to membrane contamination. Based on cytochrome oxidase and 5′-nucleotidase specific activities, the contamination figures obtained (5) were at most only 0.3% by mitochondrial membranes and 6% by synaptosomal membranes. Based on an experiment (5) in which radioiodinated mitochondria or myelin or synaptic vesicle fractions were mixed with the homogenate from which the PSD was subsequently isolated, it was found that the PSD fraction was contaminated by 3.7% of synaptic vesicle, 0.1% of myelin, and 6.5% of mitochondrial proteins. Relevant to the data of this paper, it was furthermore found using this iodination method (5) that any synaptic vesicle protein contamination of the PSD fraction was confined largely to the ~150,000 mol wt region of the PSD gel profile, with possibly some contamination in the 51,000, 45,000, and 31,000 region, but not in the 73,000 and 68,000 region of the gel where Proteins Ia and Ib appear. This region of the synaptic vesicle gel contained radioiodinated proteins, but the same region of the PSD gel did not. Finally, because of the high specific content of Proteins Ia and Ib in the synaptic vesicle fraction, and because of the possibility that Proteins Ia and Ib in the PSD fraction were there as a result of synaptic vesicle protein contamination, another type of mixing experiment was performed. The crude synaptic vesicle fraction was incubated with radioactive ATP and cAMP to label Proteins Ia and Ib, and this labeling was verified by autoradiograms of the gels. This phosphorylated fraction was mixed with an unlabeled synaptosome fraction just before lysis. From this lysed fraction, the PSD fraction was isolated, as well as a crude synaptic vesicle fraction, as described under Materials and Methods. It was found that during the lysis and centrifugation procedures there was a great loss of fraction-bound radioactivity, possibly due to protein phosphatase activity; thus, the specific radioactivity of the re-isolated crude synaptic vesicle fraction was only 3% of that of the originally added crude synaptic vesicle fraction. Autoradiograms made of the gels of these fractions showed that while the re-isolated crude synaptic vesicle preparation showed phosphorylation in the Proteins Ia and Ib region, and was similar to the original synaptic vesicle fraction (except for a great decrease in radioactivity), in the case of the PSD, there appeared a slight phosphorylation in the 51,000 mol wt region and none at all in the Proteins Ia and Ib region of the gel. Thus, it does not appear that Proteins Ia and Ib in the PSD could have been contaminants from the synaptic vesicles.

Somewhat less pure is the synaptic membrane fraction, obtained by lysis of the synaptosomes and subsequent density-gradient or flotation sedimentation, with purities in the 70–80% range as judged by some of the same criteria as above (10, 19, 26, 30, 39). Since all the fractionation procedures, including ours, are modeled on the original methods of Whittaker et al. (43) and of De Robertis et al. (9), with some modifications, our fractions are comparable to those obtained by other investigators, except as noted below for the PSD fraction.

In the various fractionation experiments, recov-

eries of total protein and of Proteins Ia and Ib were also calculated. In the case of the purified synaptic vesicle fraction 1, recoveries calculated with respect to the whole homogenate were 0.09% for total protein and 1.5% and 2.9% for Proteins Ia and Ib, and recoveries calculated with respect to the synaptosome fraction were 1.0, 11, and 12%. In the case of the PSD fraction, recoveries calculated with respect to the whole homogenate were 0.06% for total protein and 0.23 and 0.43% for Proteins Ia and Ib, and recoveries calculated with respect to the synaptosome fraction were 0.7, 1.7, and 1.8%. A principal reason for the low recoveries from whole homogenate of total protein and of Protein I content in the synaptic vesicle and PSD fractions was that only the middle parts of the bands containing these and previous fractions were used, and that the remainder of the bands and of the "intra-band" material were discarded. Thus, no attempt was made to obtain an overall 100% recovery from whole homogenate of the proteins of the various fractions. Another reason for the low recovery of PSDs was the "stickiness" of this fraction, as mentioned earlier (5). Unfortunately, at present there are no good enzymatic markers for either the synaptic vesicle or PSD fractions, so that we cannot state how much of either the synaptic vesicles or PSDs in the original homogenate or in the synaptosomes is recovered in the respective purified fractions.

The results of the present study indicate that Proteins Ia and Ib are concentrated most highly in synaptic vesicles and to a lesser extent in postsynaptic densities. Similar results have been obtained in immunocytochemical studies of the localization of Protein I (F. E. Bloom, E. Battenberg, T. Ueda, and P. Greengard, manuscript in preparation). In this latter study, peroxidase-labeled rabbit anti-Protein I antibody, prepared against Protein I purified to apparent homogeneity from bovine brain, was used to locate Protein I in rat brain. After development, the most intensely staining reaction product was found surrounding synaptic vesicles in some, but not all, nerve terminals. In those terminals showing the synaptic vesicle localization, reaction product was also observed over postsynaptic densities, although this reaction product was weaker than that associated with the synaptic vesicles.

Ng and Matus (32) have found, in a Triton X-100-derived PSD preparation similar to ours, that there were proteins whose phosphorylation was increased by cAMP, but it is difficult to correlate these with Proteins Ia and Ib. Kelly et al. (21) have recently reported that in a synaptic junction fraction there were two proteins of molecular weights 85,000 and 82,000 whose in vitro phosphorylation was increased by cAMP, and which they considered to correspond to Proteins Ia and Ib; however, these two proteins are not extracted by EDTA and EGTA from the synaptic junction fraction. These two proteins, as well as a protein kinase, were not found in a PSD fraction. However, their PSD was isolated by a *N*-lauroyl sarcosinate treatment of the synaptic membrane fraction, in contrast with our use of Triton X-100. We feel that our Triton X-100-derived PSD fraction more nearly corresponds to the in vivo situation with regard to morphology, as pointed out by Matus and Taff-Jones (28). In addition, our PSD preparation contains calmodulin (13), in contrast to the sarcosinate-derived preparation where no band corresponding to the 18,000 mol wt protein is visible (21); a confirmation of the biochemical localization of calmodulin is the observation by J. Wood, R. Cheung, and R. Wallace of the University of Tennessee of the immunocytochemical localization of calmodulin at the PSD (personal communication). Finally, in an abstract (1) it has been pointed out that in vitro incubation with radioactive ATP of a sarcosinate-derived PSD preparation gave no phosphorylated proteins whereas an in vivo infusion into the brain of $^{32}P_i$ resulted in protein phosphorylation of a subsequently isolated sarcosinate-derived PSD preparation. All these results indicate that the N-lauroyl sarcosinate treatment solubilized some PSD proteins, and, as indicated in the latter case, one of these could be the PSD protein kinase and possibly also Proteins Ia and Ib.

The presence of Protein I in synaptic vesicles as well as in postsynaptic densities suggests that Protein I has both presynaptic and postsynaptic physiological roles in nerve cells.

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