Isolation and Characterization of Postsynaptic Densities from Various Brain Regions: Enrichment of Different Types of Postsynaptic Densities

RICHARD K. CARLIN, DENNIS J. GRAB, ROCHELLE S. COHEN, and PHILIP SIEKEVITZ
Department of Cell Biology, The Rockefeller University, New York 10021, and Department of Anatomy, The University of Illinois at the Medical Center, Chicago, Illinois 60680

ABSTRACT Postsynaptic densities (PSDs) have been isolated from cerebral cortex, midbrain, cerebellum, and brain stem by the Triton X-100 method previously used in the isolation of cerebral PSDs (Cohen et al., 1977, *J. Cell Biol.* 74:181). These PSDs have been compared in protein composition, protein phosphorylation, and morphology. Thin-section electron microscopy revealed that cerebral cortex and midbrain PSDs were identical, being ~57 nm thick and composed of apparent aggregates 20–30 nm in diameter. Isolated cerebellar PSDs appeared thinner (33 nm) than cerebral cortex PSDs and lacked the apparent 20- to 30-nm aggregates, but had a latticelike structure. In unidirectional and rotary-shadowed replicas, the cerebrum and midbrain PSDs were circular in shape with a large central perforation or hole in the center of them. Cerebellum PSDs did not have a large perforation, but did have numerous smaller perforations in a lattice like structure. Filaments (6–9 nm) were observed connecting possible 20- to 30-nm aggregates in cerebrum PSDs and were also observed radiating from one side of the PSD. Both cerebral cortex and midbrain PSDs exhibited identical protein patterns on SDS gel electrophoresis. In comparison, cerebellar PSDs (*a*) lacked the major 51,000 M_r protein, (*b*) contained two times less calmodulin, and (*c*) contained a unique protein at 73,000 M_r. Calcium plus calmodulin stimulated the phosphorylation of the 51,000 and 62,000 M_r bands in both cerebral cortex and midbrain PSDs. In cerebellar PSDs, only the 58,000 and 62,000 M_r bands were phosphorylated. In the PSDs from all brain regions, cAMP stimulated the phosphorylation of Protein Ia (73,000 M_r), Protein Ib (68,000 M_r), and a 60,000 M_r protein, although cerebrum and midbrain PSDs contained very much higher levels of phosphorylated protein than did the cerebellum.

On the basis of the morphological criteria, it is possible that PSDs isolated from cerebrum and midbrain were derived from the Gray type I, or asymmetric, synapses, whereas cerebellum PSDs were derived from the Gray type II, or symmetric, synapses, Since there is some evidence that the type I synapses are involved in excitatory mechanisms while he type II are involved in inhibitory mechanisms, the role of the PSD and of some of its proteins in these synaptic responses is discussed.

Synapses in the central nervous system display on the internal surface of the postsynaptic membrane a prominent structure called the postsynaptic density (PSD). This is a disk-shaped structure in which, as has previously been shown, there is a large perforation or hole (7, 41). The PSD has recently been isolated from cerebral cortex with the use of Triton X-100 (1, 8), and this preparation was found to consist of some 15 major and at least 20 minor proteins (1). Electron micrographs of this preparation revealed cup-shaped structures ~400 nm long and ~40 nm wide, made up of apparent particles 13–28 nm in diameter (1, 8). PSDs have also been isolated through the use of deoxycholate (33) and Sarkosyl (Geigy Chemical Corp., Ardsley, N. Y.) (10), but, as pointed out by Matus and Taff-Jones (32), the preparation obtained using Triton X-100 seems

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to resemble more the *in situ* counterpart. Studies on PSDs isolated by the Triton X-100 method show the definitive presence of actin (1), calmodulin (19), a cAMP phosphodiesterase that is activated by calmodulin (20), a cAMP-dependent protein kinase and two proteins that are the substrates for this kinase (48), a calmodulin-activatable protein kinase and proteins which are substrates for this kinase (18, 20), and a major unknown protein of ~51,000 mol wt (5, 26); in addition there are indications that tubulin (1, 35) and neurofilament protein (1) are present, but the latter is probably a contaminant (11, 17, 20, 31, 37).

In this study, the Triton X-100 method of isolation was used to isolate PSDs from various general brain regions, to observe whether any differences existed among them, and thus gain clues to the possible function of the PSD in synaptic transmission, a function unknown at present. PSDs from cerebral cortex, midbrain, and cerebellum were compared by electron microscope morphology, by protein composition, and by protein phosphorylation. These comparisons showed that cerebrum and midbrain PSDs differ from cerebellar PSDs, and these differences have provided some ideas as to the function of the PSD.

MATERIALS AND METHODS

Isolation of PSDs

The procedure used to isolate PSDs from the different brain regions is a modification of the procedure used by Cohen et al. (8) in the case of cerebral cortex and is outlined in Fig. 1. The brain was removed from a dog after nembutal death, dissected into four parts, then rinsed in solution A. The cerebrum was considered as all the cortex in the forebrain, the midbrain consisted of all structures lying below the corpus callosum and above the pons, the brain stem was all material below and including the pons, and the cerebellum consisted of material above the cerebellar peduncles. Homogenization was performed by 12 up and down strokes with a motor-operated Teflon-glass homogenizer (0.25 mm clearance), using 10 g (wet weight) brain part aliquots/40 ml of solution A (Fig. 1). The resultant homogenates were combined and diluted to 10% (wt/vol) in solution A. All the *g* values are average centrifugal forces. A low-speed (1,400 *g*) pellet was obtained and washed by resuspending the pellet with three strokes of the homogenizer in the same 10% volume of solution A. The second centrifugation (710 *g*) was carried out for 10 min. After centrifugation the supernates were pooled and centrifuged at 13,800 *g* for 10 min. The resulting pellet, containing synaptosomes and mitochondria, was resuspended with six strokes of the homogenizer in solution B (Fig. 1) using 24 ml/10 g starting tissue. The sucrose gradients, using the Spinco SW 27 rotor, (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) contained 8 ml of the resuspended material, 10 ml each of 0.85, 1.0, and 1.2 M sucrose solutions all containing 1.0 mM $NaHCO_3$; these gradients were then run for 2 h at 82,500 *g*. The band between 1.0 and 1.2 M sucrose was removed by first aspirating off the solution above the band, then removing the band with a 5-ml pipette gun with a plastic tip; it was then diluted with solution B (60 ml/10 g initial wt), an equal volume of 1% (vol/vol) Triton X-100 in 0.32 M sucrose-12 mM Tris-HCl (pH 8.1) was added, and the resultant, somewhat clarified suspension was stirred in the cold for 15 min. This suspension was spun down at 32,800 *g* and the pellet was resuspended in 2.5 ml of solution B/10 g original wet weight brain part, and 2 ml of this material was layered on gradients in polyallomer tubes composed of 4 ml of 2.0 M sucrose, 3.0 ml of 1.5 M sucrose-1 mM $NaHCO_3$, and 3.0 ml of 1.0 M sucrose-1 mM $NaHCO_3$. The gradients were spun for 2 h at 201,800 *g* in the SW 40 rotor (Beckman Instruments, Inc., Spinco Div., L5-50 centrifuge). The PSDs banded between 1.5 and 2.0 M sucrose. The material above the band was removed by aspiration and the band was removed with a plastic pipette. The pipetted band was diluted to a final volume of 6.0 ml with solution B, and an equal volume of 1% Triton-150 mM KCl was added. This suspension was spun for 20 min at 201,800 *g* in the SW 40 rotor. The resultant pellet was resuspended by homogenization with a motor-driven Telfon-glass homogenizer. The yields were (mg/10 g initial wet wt tissue): cerebrum, 2–3; midbrain, 1.5–2; cerebellum, 1.

Gel Electrophoresis

Gel electrophoresis was performed as described before (8).

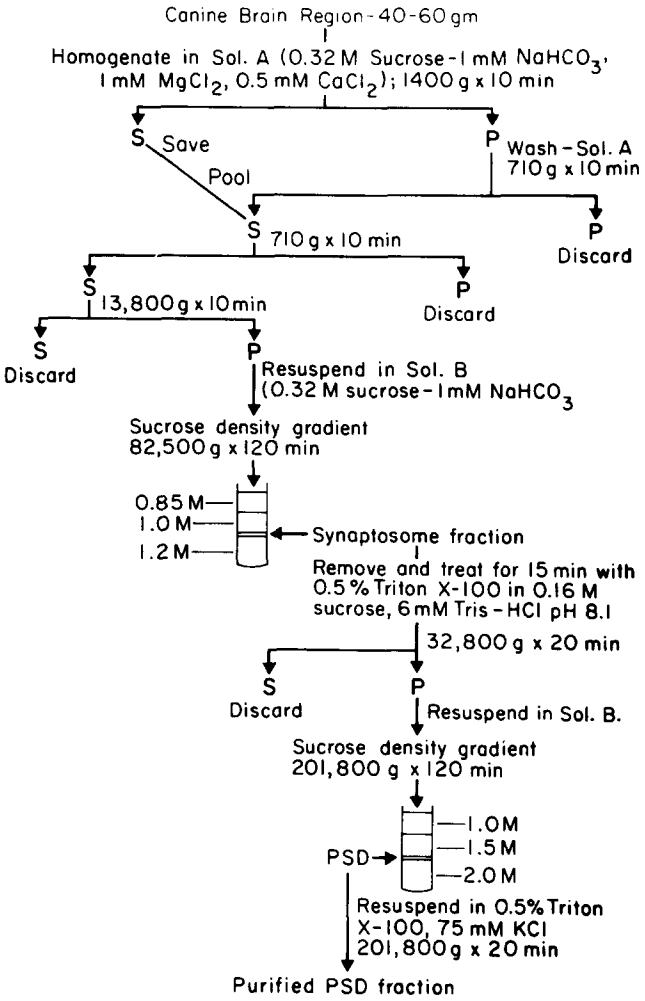

FIGURE 1 Fractionation scheme for the preparation of PSDs from various brain regions. Discussed more fully in Materials and Methods.

Electron Microscopy

Electron microscopy, both thin section and replicas, was performed as described before (8). In addition, rotary shadowing was done at an angle of 20° with platinum-carbon for 6 s while the specimen was rotated six times. The replica was then coated at an angle of 90° with carbon for 6 s.

Protein Phosphorylation

Calcium-dependent protein kinase activity of the PSD was performed according to the method of Schulman and Greengard (45). The standard reaction mixture contained in a final volume of 0.1 ml: 50 mM PIPES buffer, pH 7.0; 10 mM $MgCl_2$; 1 mM dithiothreitol (DTT); 0.2 mM EGTA; plus 50–100 μg PSD protein. The plus calcium samples also contained 0.5 mM $CaCl_2$ with or without 3 μg purified canine brain calmodulin (prepared by the method of Watterson et al. (53). After a 1-min preincubation at 30°C, 5 μM ATP (5–10 μCi γ-[^{32}P]ATP) was added and the mixture was further incubated at 30°C. The reaction was terminated by the addition of 2% SDS, and SDS PAGE was performed as described in Materials and Methods. Autoradiography was performed on the dried gels using Cronex 2 DC Medical Film (DuPont Instruments, Wilmington, Del.). The cAMP protein kinase activity of the PSD was performed as described above, except that 10 μM cAMP was used in place of the calcium and calmodulin. Isobutylmethylxanthine (1 mM) was also added to inhibit the breakdown of cAMP (48, 49).

RESULTS

Morphology

Figs. 2–4 show representative thin-section views, while Figs.

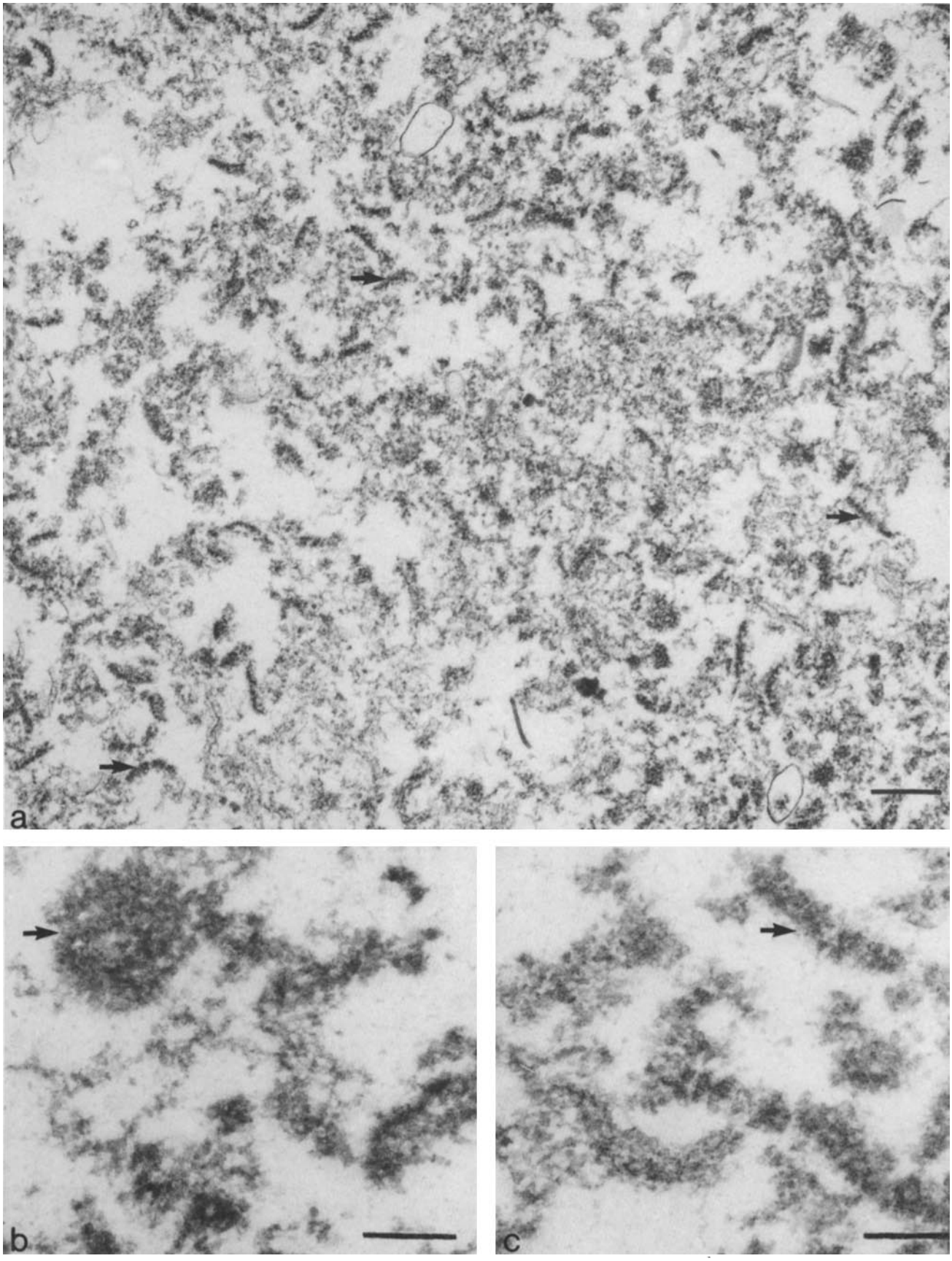

FIGURE 2 Electron micrographic examination of a PSD preparation isolated from cerebral cortex. (*a*) Large field view of preparation. Arrows depict PSDs. The nondescript material is probably PSDs sectioned in different planes, plus filamentous material attached to the cytoplasmic side of the PSD, plus a small amount of contaminants from other cellular structures (cf. Cohen et al. [8]). Bar, 500 nm. × 25,000. (*b*) *En face* view of PSD (arrow), with a perforation in the middle of the disk. Bar, 200 nm. × 82,500. (*c*) Cross-sectional view of the PSD (arrow). Bar, 200 nm. × 82,500.

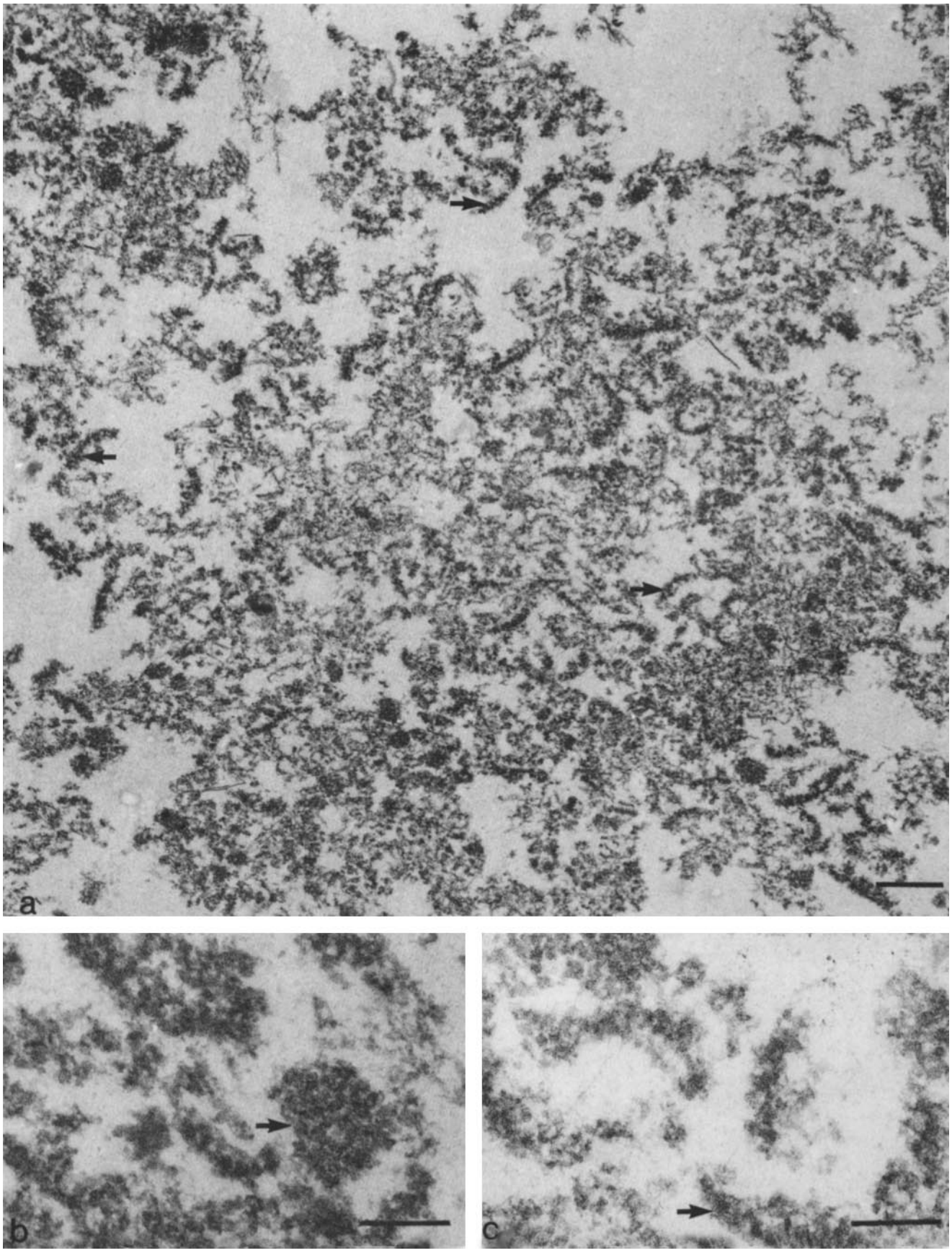

FIGURE 3 Electron micrographic examination of a PSD preparation isolated from midbrain. (*a*) Large field view of preparation. Arrows indicate typical PSDs; description of material as given in Fig. 2. Bar, 500 nm. × 25,000. (*b*) *En face* view of PSD (arrow) with possible perforation in the center. Bar, 200 nm. × 82,500. (*c*) Cross-sectional view of the PSD (arrow). Bar, 200 nm. × 82,500.

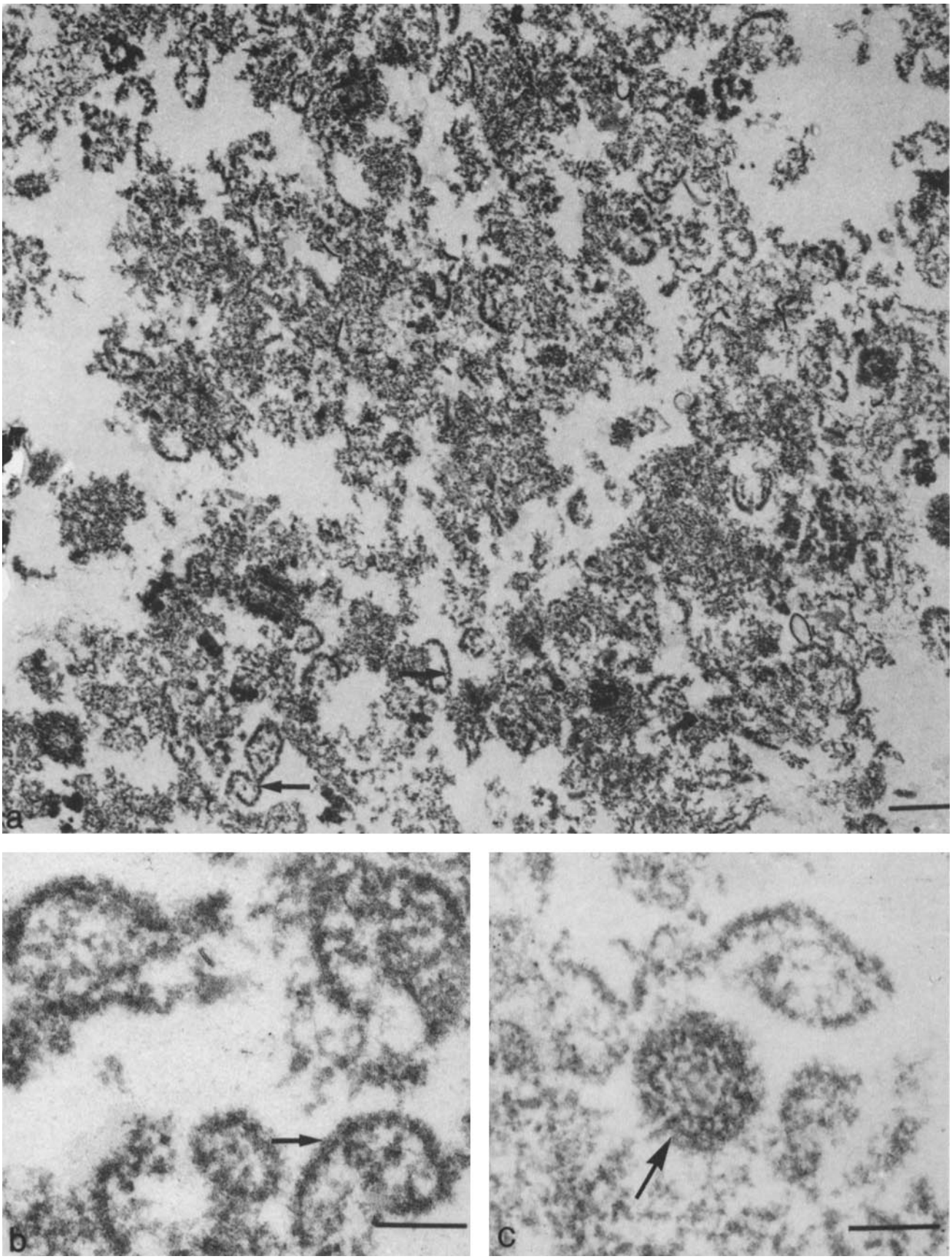

FIGURE 4 Electron micrographic examination of a PSD preparation isolated from cerebellum. (*a*) Large field view of preparation. Arrows indicate typical PSDs; description of other material as given in Fig. 2. Bar, 500 nm. × 25,000. (*b*) Cross-sectional view of PSD (arrow) indicating unusual thinness of these PSDs. Bar, 200 nm. × 82,500. (*c*) *En face* view of the PSD (arrow) indicating the latticelike nature of the structure. Bar, 200 nm. × 82,500.

5 and 6 show replica views, of PSD preparations isolated from various brains parts. Although the cerebrum and midbrain PSD preparations are indistinguishable from one another, those from cerebellum and brain stem present differences. Fig. 2*a* is a low-magnification field showing the morphology of the PSD preparation isolated from cerebral cortex. A number of PSDs can be seen in this preparation (arrows). A detailed morphological description of this preparation has been given earlier (8). The nondescript material is probably PSDs sectioned in different planes, plus filamentous material attached to the cytoplasmic side of the PSD, plus a small amount of contaminants from other cellular structures (8). There is a small amount of vesicular contaminants in the preparation. When viewed from the side (Fig. 2*c*), the PSDs are flat to semicircular, with an average thickness of 58 nm, based on 50 measurements. An *en face* view (Fig. 2 b) shows a disk-shaped structure with a less dense center, as has been previously described (7, 41). Both the *en face* and cross-sectional views of the PSD show the presence of 20- to 30-nm particle-like aggregates. These have been previously described (1, 8, 35).

The PSD preparation from midbrain (Fig. 3*a*) shows a preparation similar to that of cortex, wth size and shape of the PSD being the same. In a cross-sectional view (Fig. 3*c*), the thickness of the PSD averaged 56 nm, based on 50 measurements, not significantly different from that of the PSD from cerebral cortex. An *en face* view (Fig. 3*b*) shows an almost identical appearance as PSDs isolated from cerebral cortex, that is, a disk-shaped structure with 20- to 30-nm aggregates and a less dense center.

The cerebellar PSD preparation is shown in Fig. 4*a*. The PSDs in this preparation are much thinner than those from cerebral cortex or midbrain, the average cross-sectional thickness being 33 nm, based on 50 measurements, about half the thickness of cerebral cortex or midbrain PSDs (compare Fig. 4*b* with Figs. 2*c* and 3*c*). Other features of these PSDs are the high degree of convexity within each PSD (Fig. 4*b*), and the large amount of subsynaptic web material associated with them (Fig. 4*b*). The functional significance of both of these observations is unknown at present. *En face* (Fig. 4*c*) the PSDs are disk shaped, but apparently lack the 20- to 30-nm aggregates present in cerebrum PSDs. In their place is a latticelike structure (Fig. 4*c*) that is similar to cerebrum PSDs treated with sodium deoxycholate to produce what has been named a PSD lattice (32). However, the sodium deoxycholate-produced lattice from cerebral cortex PSDs retains the original thickness of a cerebrum PSD.

The PSD preparation from brain stem represented a much more variegated picture (not shown) than those from other brain areas. Though there were recognizable PSDs present, with a shape and thickness similar to that of cerebral cortex PSDs, there was much more unrecognizable material plus much filamentous material that was probably intermediate-filament contamination (20), as evidenced by a comparison with filaments isolated by the method of Liem et al. (31). Because of the low degree of purity and low yield of the preparation, little can be written now concerning this preparation.

It has been possible to verify the difference in thickness between PSDs isolated from cerebral cortex and those isolated from cerebellum, as indicated by the replica pictures of each of these preparations (Fig. 5). The unidirectional shadowing makes it evident that the majority of the PSD structures from cerebral cortex (Fig. 5*a*) are much thicker than those from cerebellum (Fig. 5*b*). Also very evident is that although most of the thicker PSDs have an apparent hole in the center (arrows, Fig. 5*a* and *b*), none of the thinner ones do (Fig. 5*b*). Rotary shadowing of these replica preparations brings out other features of the isolated PSDs. Those from cerebral cortex or midbrain (Fig. 6*a*) show, in addition to the obvious large central hole, the particulate nature of the PSD disk made up of apparently 20- to 30-nm-diameter aggregates. In contrast, PSDs isolated from cerebellum (Fig. 6*b*) do not show a large central hole but, instead, many smaller perforations; in addition the particulate nature of the disk is much less apparent than in the case of the cerebral or midbrain PSD, with a latticelike structure very predominant. Whether these 20- to 30-nm-diameter particles are the "extra materials" in the cerebral PSDs which makes them thicker than PSDs from cerebellum is not clear. In some cases, a preparation shows many broken-up PSDs, which appear "opened up" (Fig. 6*c*). Under these circumstances, one begins to observe 6- to 9-nm diameter filaments connecting the 20- to 30-nm diameter particles (Fig. 6*c*); these may correspond to actin filaments holding the PSD particles together, for actin is found in these preparations (1, 26). Fig. 6*c* also shows what are probably intermediate filaments that may be attached to the PSD but are most likely a contaminant in the preparations. The 6- to 9-nm-diameter filaments also frequently extend from one side of the thick PSDs (Fig. 6*d*). Whether this is part of the subsynaptic web or part of the cell cytoskeleton is unknown. However, the subsynaptic web of cerebellum PSDs seen in thin sections has never been observed in shadowed preparations, for these PSDs are viewed *en face* and tend to flatten into symmetrical disks.

Protein Composition

Fig. 7 shows two examples of the SDS PAGE profiles of the proteins of PSD preparations from different brain parts. The PSD preparations from cerebrum and midbrain have nearly identical protein compositions. The major protein of the cerebrum and midbrain PSD, first thought to be tubulin (15, 52) and later to be neurofilament protein (8, 56), is an unknown protein at 51,000 M_r (cf. reference 26), hereafter referred to as the major 51,000 M_r protein. It can be separated by SDS PAGE under certain conditions from the 50,000 M_r intermediate-filament band (20). A previous description of the proteins from the cerebral cortex preparation and a partial (tubulin) or complete (actin) identification of some of them have been given by us (1) and by others for tubulin (26, 36, 52) and for actin (26). Recently, another of the major proteins, the one at 18,000 M_r, has been identified as calmodulin by biochemical (19) and immunochemical (55) means. Of the minor proteins, protein I (consisting of proteins Ia and Ib, both substrates for a cAMP-dependent protein kinase) has been identified by biochemical (48) and immunological (2) means as being part of the PSD. Of all the major proteins, only the myelin basic protein (8) and probably the intermediate-filament proteins (20, and A. J. Matus, personal communication) are contaminants in these preparations.

Cerebellar PSDs present a different composition. Whereas the general protein profile is similar to that of cerebral PSDs, the amounts of the major 51,000 M_r protein and of calmodulin are greatly reduced, and a protein unique to cerebellar PSDs is present at ~74,000 M_r.

A brain stem preparation presents a more altered profile. This preparation lacks the major 51,000 M_r protein and con-

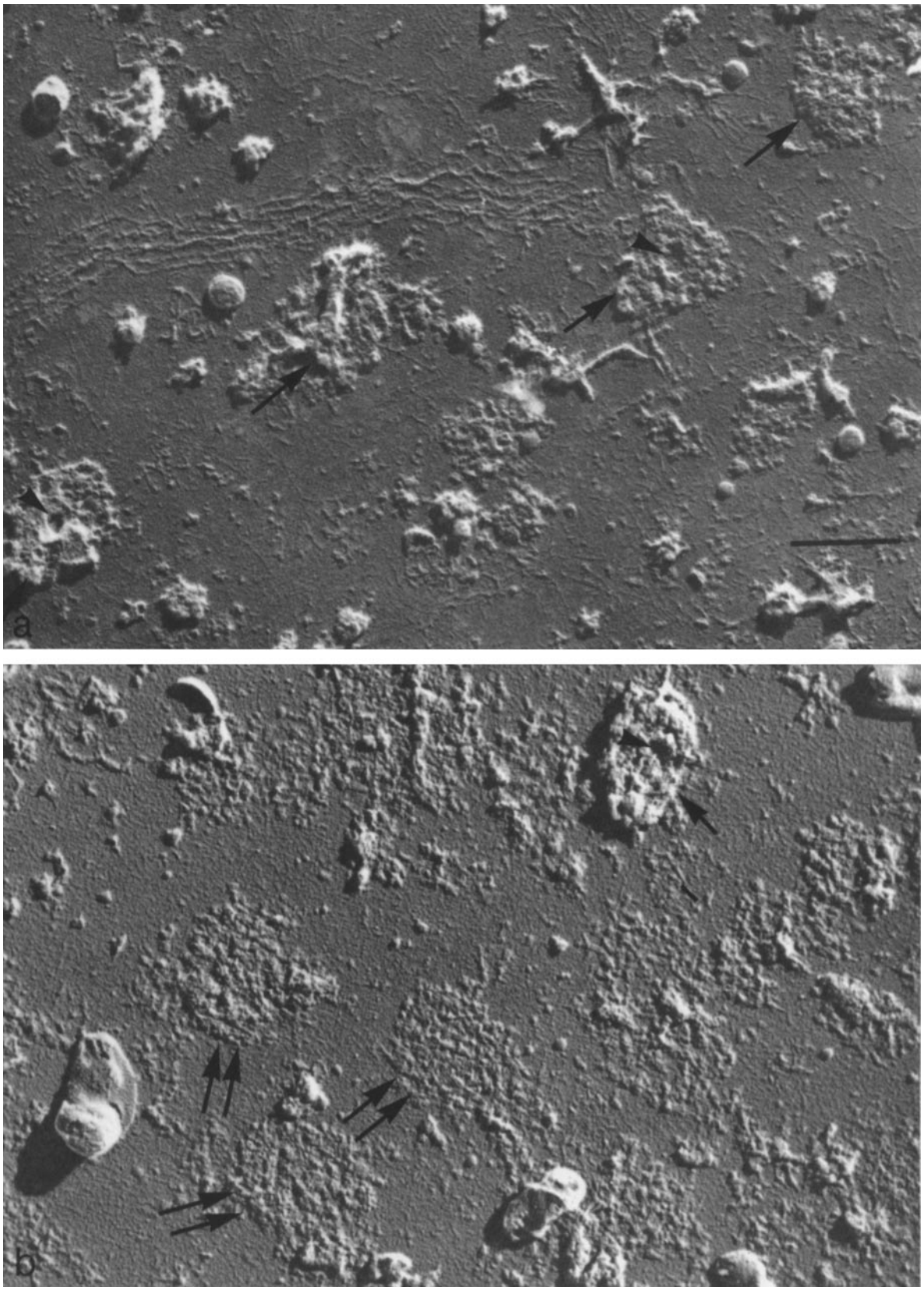

FIGURE 5 Isolated PSDs from cerebral cortex (*a*) and from cerebellum (*b*) as viewed by the replica method, with unidirectional shadowing. Single arrows point to thick PSDs, double arrows point to thin PSDs, and arrowheads point to apparent holes in the thick PSDs. Note contaminating filaments in the cerebral cortex PSD preparation (*a*). The PSD from cerebral cortex (second arrow from the left) appears to be half-turned upwards. There is an apparent hole at the fold. Bar, 500 nm. × 36,000.

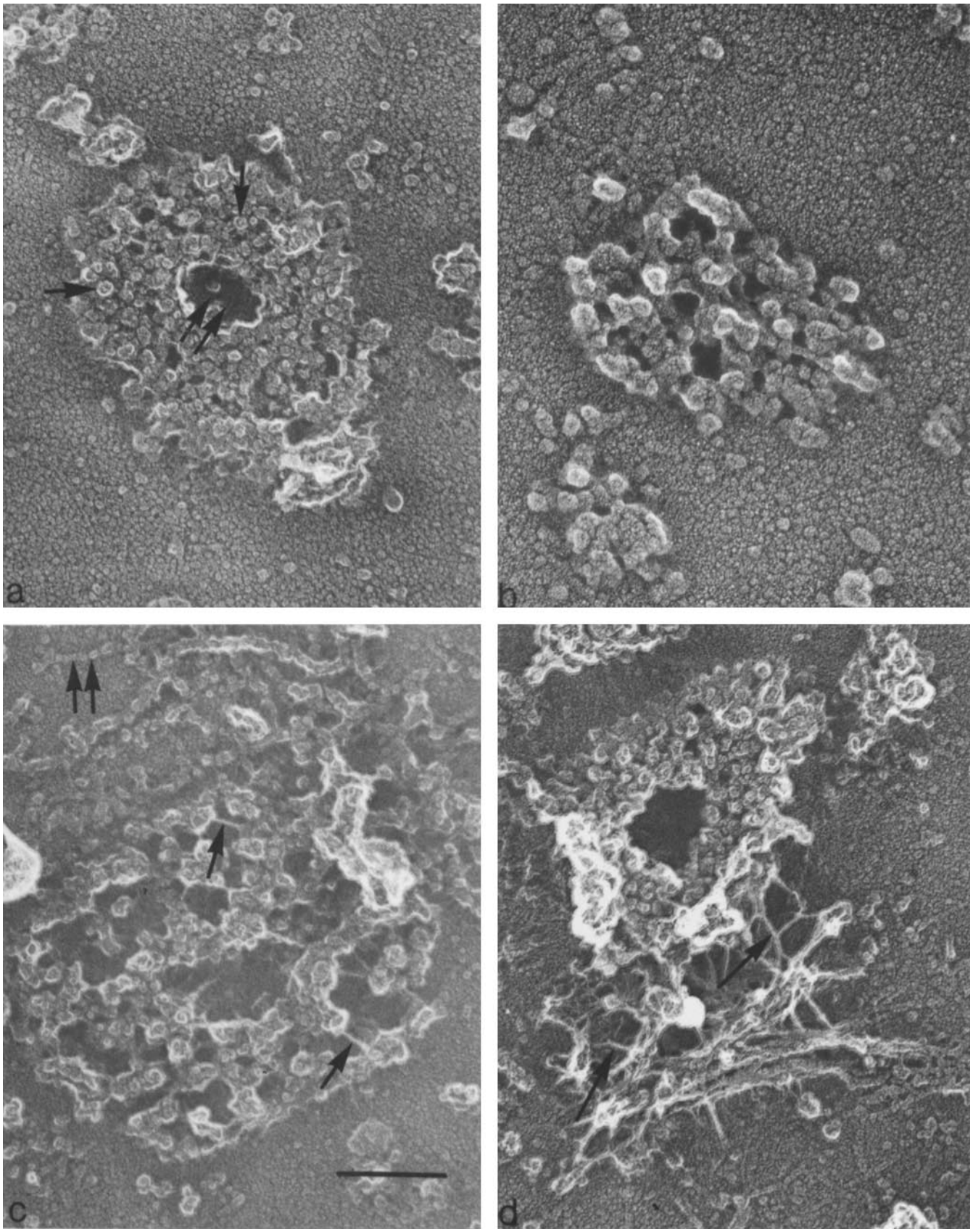

FIGURE 6 Isolated PSDs from midbrain (*a*), cerebellum (*b*), and cerebral cortex (*c* and *d*) as viewed in high magnification by the replica method with rotary shadowing. In a, single arrows point to possible particulate bodies, and the double arrows point to the large central hole. In *c*, the PSD is apparently broken up; the single arrows point to 6- to 9-nm filaments and the double arrows point to 12- to 15-nm filaments. *d* Shows a PSD with extensions of 6- to 9-nm filaments (arrows) apparently arising from one surface of the PSD. Bar, 200 nm. × 100,000.

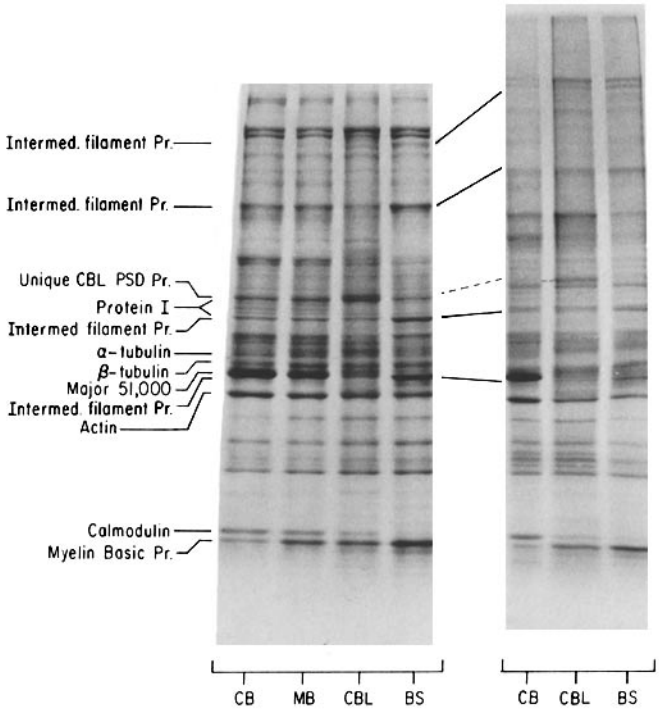

FIGURE 7 SDS PAGE profile of proteins of PSDs from different brain parts. Equal amounts (100 μg protein) from each preparation were run on the gels. The markers on the left indicate the positions of known proteins in the PSD preparation (cf. text); of these only the myelin basic protein and the intermediate filament proteins are believed to be contaminants (cf. text). The two photographs represent two different isolations, with the solid lines between them showing the positions of the intermediate filament bands and with the dotted line indicating the position of a protein unique to the cerebellar PSD preparation. The two brain stem protein profiles indicate the high variability of intermediate filament content. The cerebellum PSD preparation shows a reduced content of the 51,000 M_r protein and calmodulin, while the brain stem preparation almost completely lacks the bands corresponding to these two proteins. The profiles of the cerebral cortex and midbrain PSD preparations are remarkably similar. *CB*, cerebrum; *MB*, midbrain; *CBL*, cerebellum; *BS*, brain stem.

tains a very small amount of calmodulin. However, as this preparation is isolated in very low yield and is usually heavily contaminated with intermediate filaments (Fig. 7), we cannot draw any definitive conclusions on the structure or protein composition of brain stem PSDs.

Protein Phosphorylation

The results of phosphorylations by ATP of PSD proteins from different brain parts in the absence or presence of cAMP and of calcium and calmodulin are shown in Figs. 8 and 9. Fig. 8 again illustrates the similarity in protein composition between PSDs from cerebral cortex and midbrain, and the differences between these and those from cerebellum. In PSDs from cerebrum, cerebellum, and midbrain, the addition of cAMP to the phosphorylation mixture resulted in the reproducible increased phosphorylation of three proteins: Protein Ia (73,000 M_r), protein Ib (68,000 M_r), and a minor protein at ~57,000 M_r (Fig. 9). The presence of proteins Ia and Ib in the cerebral cortex PSD has been noted before, by biochemical methods on an isolated PSD preparation (48) and by immunochemical methods *in situ* (2). This phosphorylation of proteins Ia and Ib occurred in all cases whether a purified protein

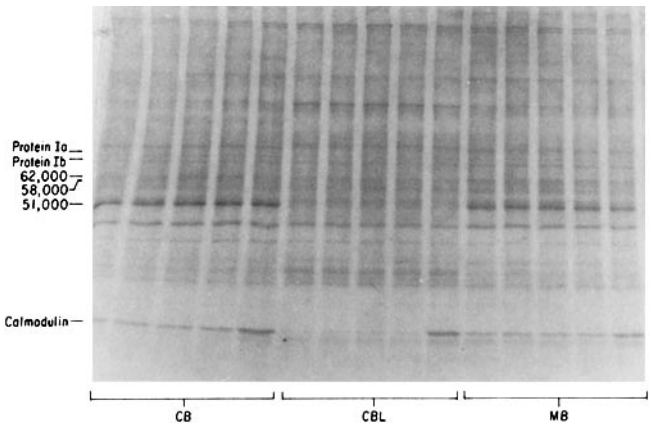

FIGURE 8 SDS PAGE profile of proteins of PSDs isolated from various parts of the brain. Equal amounts (50 μg protein) from each preparation were run on the gel. The comparative autoradiography is shown in Fig. 9, while the markers on the left indicate the positions of the major phosphorylated proteins shown in Fig. 9. Again, note the marked reduction in the bands corresponding to the major 51,000 M_r and calmodulin bands in the cerebellar preparations. The intense band in the calmodulin position in the fifth gel of each series is caused by the addition of exogenous calmodulin (cf. Fig. 9). *CB*, cerebrum; *CBL*, cerebellum; *MB*, midbrain.

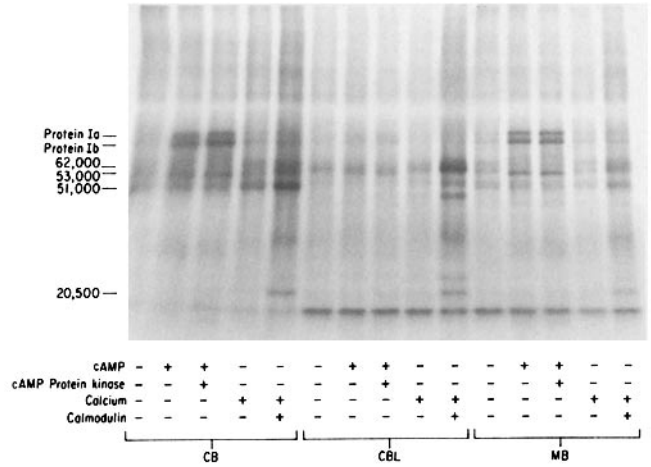

FIGURE 9 SDS PAGE autoradiographic profile of proteins of PSDs isolated from various parts of the brain. The gel shown in Fig. 8 was prepared for the autoradiography shown in this figure. The markers on the left indicate the positions of those proteins whose phosphorylation was increased the most by the additions given on the bottom. The incubation conditions are given in Materials and Methods. The band that is uniformly phosphorylated under all conditions in all samples near the bottom of the gel (M_r = 17,000) is the myelin basic protein. *CB*, cerebrum; *CBL*, cerebellum; *MB*, midbrain.

kinase was added or not (Fig. 9), implying that a cAMP-dependent protein kinase, fully accessible to the substrate protein and to cAMP, is located in the PSD in these preparations. This had been noted before in the case of the cerebral cortex PSD preparation (48). The amounts of protein I in cerebrum and midbrain PSDs seem to be identical (Fig. 9), as noted by their increased phosphorylation of these proteins. Previously (48), it had been found that the amount of the phosphorylations of these proteins can be correlated to the actual amounts of these proteins. In contrast, the cerebellum PSDs (Fig. 9) contain much less of proteins Ia and Ib, as indicated by their decreased phosphorylations. However, the

immunochemical results of Bloom et al. (2) gave larger amounts of protein I in various areas of the cerebellum than in cerebral cortex. The difference between the results may be explained by the observation that in both types of observation (2, 48) the amount of protein I in the PSD is much smaller than that in synaptic vesicles, and thus the smaller amounts of protein I in the PSDs from cerebellum as compared to cerebral cortex may be masked by the much higher amounts in the synaptic vesicles in both cases. In another study (43) in which a comparison was made of cAMP-dependent phosphorylation from different brain parts, it was found that synaptic membrane preparations from cerebrum, midbrain, and cerebellum all had the same amount of proteins believed to be identical to proteins Ia and Ib. However, De Blas et al. (12), using a total particulate fraction from various brain areas, did find two phosphorylated proteins which they believed to be analogous to Proteins Ia and Ib to occur in much higher amounts in cerebrum than in cerebellum, a result in agreement with ours on isolated PSD preparations.

Previously, a Ca^{++}-calmodulin-stimulated protein phosphorylation has been noted in a cerebral cortex synaptic membrane fraction (45) and in a cerebral cortex PSD preparation (18, 20). This result has not been extended to studies on PSDs from other brain areas. Unlike that observed by the addition of cAMP, calcium stimulation in all cases was not fully activated unless exogenous calmodulin was also added (Fig. 9). However, if the PSD is frozen or sonicated first, the calcium-stimulated phosphorylation is increased and occasionally equals that with added Ca^{++} plus calmodulin (18, 20). This suggests that calmodulin is not directly accessible to the protein kinase but is within close proximity. In fresh PSDs, calcium plus calmodulin reproducibly and identically stimulated greatly the phosphorylation of the major 51,000 and 62,000 M_r proteins in the cerebrum and midbrain PSD preparations (Fig. 9). In some cases, a 58,000 M_r protein and a 20,500 M_r protein were also increased by the addition of Ca^{++} plus calmodulin (Fig. 9), while in other cases this was not so apparent (data not shown). Cerebellar PSDs show a decreased phosphorylation of the 51,000 M_r protein (Fig. 9) consistent with the marked decrease in the amount of this protein in these PSDs (Figs. 7 and 8), but the increase in the phosphorylation of the 62,000 M_r band seemed to be equal to those of cerebrum PSDs (Fig. 9). In addition, the major protein phosphorylated in the cerebellum PSDs was one at 58,000 M_r (Fig. 9), with a protein at ~48,000 M_r being uniquely phosphorylated in these PSDs (Fig. 9).

DISCUSSION

Morphology: PSDs from Type I and Type II Synapses

Table I gives a summary of the morphological characterisitcs of PSDs isolated from the various brain parts. The different thicknesses of PSDs isolated from cerebrum and midbrain as compared to those from cerebellum suggest that these are derived from two different general classes of synapses. Gray (21) divided synapses into two classes: type I synapses have a much more prominent band of dense-staining material at the postsynaptic side than they do at the presynaptic side, while type II synapses have a less evident and thinner postsynaptic band. Colonnier (9) also observed this difference in synapses and labeled the first an asymmetric synapse because of the difference in prominence between the two sides of the synapse, and labeled the second type of synapse symmetric. However,

TABLE I

Morphological Characteristics of PSDs Isolated from Various Brain Regions

Characteristics	Cerebrum	Midbrain	Cerebellum
Approx. thickness, *nm*	58	56	33
Subsynaptic bodies	+	+	+
Subsynaptic web	+	+	+++
20- to 30-nm particles	+	+	−
Large central hole	+	+	−

the distinction between these two types may not be that clear cut (40, 42, 50). If we assume that there is meaning to this general classification, then we can add that because the isolated cerebellar PSDs are half as thick as PSDs from the other brain parts, it may be that the cerebellum PSD preparation represents an enriched population of PSDs from Gray type II or symmetric synapses, whereas the cerebrum and midbrain PSD preparations may represent enriched populations of PSDs from Gray type I or asymmetric synapses.

Why we obtain this increased enrichment is not known, as both of these general brain areas contain both types of synapses (cf. reference 51). Rostas et al. (44) also concluded that their PSD preparation from cerebral cortex, isolated by a method different from ours, also arose from asymmetric or type I synapses. It could be that during the isolation procedure of PSDs from cerebrum we have lost the type II synapses, with the reverse occurring in the case of the cerebellum. Though Matus and Walters (34) have concluded that a lysed synaptosomal preparation from cerebral cortex contains both type I and type II synaptic complexes, there are indications that various types of synaptosomes, based on neurotransmitter content, can be partially separated from each other (4, 13, 16, 25, 27, 38, 39, 54). Therefore, one possible reason for our enrichments of PSDs could be that we are initially differentiating the synaptosome populations of cerebrum and midbrain on the one hand from that of the cerebellum on the other. This situation has been partially verified by Israel and Whittaker (24) who found that some of the synaptosomes from cerebellum sediment at low speed; it could be that we therefore have lost type I synapses of the cerebellum in this pellet, and have obtained only type II synapses at the conventional place in the isolation scheme as outlined in Fig. 1. However, a repetition of the Israel and Whittaker experiment did not result in any recognizable PSDs obtainable from this low-speed pellet by the use of Triton X-100. Another possibility is that the later parts of the isolation procedure, particularly the detergent treatment, caused a loss of PSDs from type II synapses in cerebrum and a loss of PSDs from type I synapses in cerebellum, but this seems logically improbable. Still another possibility is that under our isolation conditions, cerebellar PSDs have lost proteins, particularly the major 51,000 M_r protein of the cerebrum/midbrain PSDs; however, this is unlikely, as the 51,000 M_r protein is highly insoluble, only going into solution by SDS addition. Whatever the reason, it seems clear from the summary given in Table I, that we have isolated PSDs which seem to come from either type I or type II synapses, from the cerebrum/midbrain and from the cerebellum respectively, and that it would be instructive to look further into their differences and similarities. However, is should be added that even in these PSD preparations, from cerebrum/midbrain and from cerebellum, there are probably populations of PSDs from different kinds of synapses, based on the neurotransmitters involved.

There are differences in morphology other than thickness between the PSDs isolated from these apparent two types of synapses. One of the major differences is in the appearance of particular aggregates. The *en face* view of the cerebrum PSD typically shows the presence of these particlelike aggregates 20–30 nm in diameter (Figs. 2 and 6*a*), which have been previously described in isolated PSDs (1, 8). In addition, Matus et al. (35) have previously described 20-nm dense staining bodies at the postsynaptic thickening of isolated synaptic junctions which arise from type I synapses. Landis and Reese (28) and Landis et al. (29), using freeze fracture, observed that presumably excitatory (type I) synapses have 8- to 13-nm bodies in the postsynaptic membrane, whereas presumably inhibitory (type II) synapses do not. And incidentally, Matus and Walters (34) have found that type II synapses have concanavalin A binding sites on the postsynaptic junction membrane, whereas type I synapses do not. Matus et al. (35) have suggested that the 20-nm aggregates mentioned above may traverse the postsynaptic membrane and be the 8- to 13-nm particles viewed in freeze-fracture studies of the postsynaptic membrane. These observations are reminiscent of an earlier suggestion by Peters and Kaiserman-Abramof (41) that 30-nm particles which they observed *in situ* in PSDs of type I synapses extended into the synaptic cleft. However, at present there is little direct evidence relating the 20- to 30-nm aggregates and the 8- to 13-nm particles. Matus and Walters (33) have described a postsynaptic lattice structure of the PSD, obtained by deoxycholate treatment of synaptosomes, which could be a framework of these 20- to 30-nm aggregates. An *en face* view of a typical thin cerebellum PSD shows a structure resembling the postsynaptic lattice (only thinner), and few apparent aggregates can be found (Figs. 4 and 6*b*).

Another distinction which was observed consistently between cerebral/midbrain PSDs and those from cerebellum was the presence of a large central perforation in the former case and not in the latter, as was easily observed in the replica preparations (Figs. 5 and 6). On the other hand, cerebellar PSDs showed many more smaller perforations, but whether this is caused by an opening-up during the isolation procedure is not known. In some cases, what is obviously an expanded, altered PSD from cerebral cortex (Fig. 6*c*) does show these perforations. The initial observation by Peters and Kaiserman-Abramof (41), verified later (7), showing a large central perforation in the PSD was made on dendritic spines, which are predominantly of the type I synapse (20), partially confirming our view that our cerebral/midbrain PSDs arise from type I synapses. It is interesting that Landis and Reese (28) have found that occasionally the collection of particles in postsynaptic membranes of asymmetric synapses are arranged in a circular ring around a nonparticle center, the dimensions of which fit the size of the hole in our isolated type I or asymmetric PSDs. The reason for this kind of perforated structure of PSD is at present unknown.

On the basis of the observations in this and previous papers, we would like to picture the thin, type II PSDs as being a multiperforated disk, composed of proteins not in the form of aggregates, some of which attach this structure to the membrane. The type I PSD retains this basic structure, but adds onto it large particulate aggregates, 20–30 nm in diameter; in terms of protein composition, the only large difference is the addition of 51,000 M_r protein and of calmodulin to the basic structure. This 51,000 M_r protein cannot be the major structural protein of the PSD, for it is almost entirely lacking in the type II PSD; indeed, the latter contains not one protein that is present in abundance over others. Because actin is found in rather large amounts in all the PSD preparations, it could be one of the cementing blocks holding the structure together, as those thin filaments seen in Fig. 6*c* connecting the particles may be actin. The addition of the superstructure of the type I PSD onto the basic type II PSD structure is accompanied by the appearance of a large central perforation through the entire PSD structure the significance of which is presently unknown. Support for this superstructure theory is provided by Hinds and Hinds (23), who concluded that during development in olfactory axodendritic synaptogenesis all asymmetrical synapses pass through an immature symmetrical synapse stage before being transformed into a recognizable asymmetrical one during a period estimated to be 9–10 h.

Protein Composition and Phosphorylation

A comparison of the protein composition of cerebrum and cerebellum PSDs (Table II) may give an insight into the mechanisms of PSD function in type I and type II synapses. The major difference in protein composition between cerebrum and cerebellum is in the amounts of the major 51,000 M_r protein and calmodulin. As there is very little 51,000 M_r protein in cerebellum, the 51,000 M_r protein may be a protein unique to asymmetric type I synapses, no matter what transmitter may

TABLE II

Summary of Protein Composition and Protein Phosphorylation of PSDs from Various Brain Regions

Proteins (M_r)	Cerebrum	Midbrain	Cerebellum
A. Relative composition of selected proteins			
74,000	−	−	+
73,000, 68,000 Protein I	+	+	±
59,000, 55,000 Tubulin subunits	++	++	++
51,000	+++	+++	±
45,000—Actin	++	++	++
18,000—Calmodulin	++	++	+
B. Increased phosphorylation upon addition of calcium plus calmodulin			
73,000, 68,000 Protein I	−	−	−
57,000	−	−	−
62,000	++	++	++
58,000	±	±	++
51,000	+++	+++	±
48,000	−	−	+
20,500*	+	+	+
C. Increased phosphorylation upon addition of cAMP			
73,000, 68,000 Protein I	+++	+++	+
57,000	+	+	±
62,000	−	−	−
58,000	−	−	−
51,000	−	−	−
20,500	−	−	−

* Variable.

be involved. This has also been inferred by Rostas et al. (44), based on the high amounts of this protein in synaptic junction complexes isolated from various cerebral and midbrain regions postulated to have asymmetric synapses. A second difference is that calmodulin is reduced by ~50% in cerebellum PSDs as compared to cerebrum PSDs. This confirms the work of Sobue et al. (46) in which they determined the concentration of calmodulin in membrane preparations from cerebrum and cerebellum. The higher concentration of calmodulin suggests that calmodulin may be very important in type I synapses but the presence of still detectable amounts of calmodulin in cerebellum PSDs does not rule out a role in these type synapses.

The phosphorylation of some of the proteins of PSDs under varying conditions may give some further insight into possible differences in function between the two classes of PSDs. Table II gives a summary of our results. In the presence of calcium plus calmodulin, cerebrum PSDs exhibit increased phosphorylation of the major 51,000 and 62,000 M_r proteins. In contrast, the cerebellum PSDs have a greatly reduced phosphorylation under these conditions in the 51,000 M_r region. As protein phosphorylation has been proposed to be important in synaptic function (22), the uniqueness of the major 51,000 M_r protein in cerebrum and midbrain PSDs may indicate that phosphorylation of this protein is involved in the generation of postsynaptic potentials in type I synapses. On the other hand, the predominance of the increased phosphorylation upon addition of Ca^{++} and calmodulin of the 58,000 M_r protein in cerebellum may indicate its possible involvement in the generation of postsynaptic potentials in type II synapses. However, the phosphorylation of the 62,000 M_r protein cannot entirely be assigned to Type II synapses as this protein is apparently present in both types of PSD. Whether this protein is involved in the action of both types of PSD or is present in the preparation of cerebrum PSDs because of the presence therein of PSDs from type II synapses, remains to be determined.

Cyclic AMP-dependent phosphorylation of cerebrum, midbrain, and cerebellum PSDs produces the same pattern. The major phosphorylated bands are in proteins Ia and Ib (48), though the degree of phosphorylation varies among the preparations. The other minor phosphorylated band (~57,000 M_r) possibly represents autophosphorylation of the regulatory subunit of the cAMP-dependent protein kinase (49). But, cerebrum PSDs have much greater phosphorylation of proteins Ia and Ib upon addition of cAMP than do cerebellum PSDs. Thus, it would appear that the cAMP-dependent phosphorylations may be involved in the function of the type I synapses and not that of the type II synapses. However, in cerebral cortex, protein I is much higher enriched in the synaptic vesicles than in the PSD, so that it also has some presynaptic role.

PSDs from Type I Excitatory and from Type II Inhibitory Synapses

The general classification of synapses as type I and type II has led Eccles (14) to propose that type I synapses mediate excitation responses and type II synapses mediate inhibitory responses, and Colonnier (9) later suggested the same correlation. This hypothesis was based on work summarized by Eccles (14) and by Walberg (51), that in the hippocampus and cerebral cortex there was a correlation between type I structure and known excitatory synapses and between type II structure and known inhibitory synapses; in the cerebellum the evidence from various sources seemed to be somewhat contradictory and thus not that clear-cut. Later work by Landis et al. (29) on synapses in the olfactory bulb and by Landis and Reese (28) on synapses in the cerebellar cortex strengthened this hypothesis. These authors could make correlations between the known excitatory and inhibitory properties of certain synapses in these regions and the prevalence of either type I or type II differentiations there. At the same time, results from many laboratories (summarized by Bodian [3] and by Uchizono [47]) have brought forth another correlation, that of the appearance of spherical synaptic vesicles in excitatory synapses and of flattened or oblong vesicles in inhibitory synapses. And indeed, the finding that the presence of spherical vesicles and of type I differentiation go together and that the presence of flattened vesicles and of type II differentiation go together has prompted Uchizono (47) to propose that excitatory synapses have a type I PSD and spherical presynaptic vesicles and inhibitory synapses have a type II PSD and flattened presynaptic vesicles.

On the basis of the above assumptions, we can summarize our results in Table III. We first postulate that our enriched population of thick PSDs from cerebral cortex and midbrain arise from type I excitatory synapses and that the enriched population of thinner PSDs from cerebellum arise from type II inhibitory synapses. If this is the case, then the difference in protein composition between these two types of PSDs takes on functional significance. Thus, the far greater amounts of the unknown 51,000 M_r protein, of calmodulin, and of the substrate proteins Ia and Ib for the cAMP-activatable protein kinase in the PSDs from cerebral cortex and midbrain over that in the PSDs from the cerebellum, would suggest that these proteins are involved in excitatory modulation of the transmission signal which occurs at these synapses. However, it must be stated that our PSD populations from these two sources are probably still a mixture of PSDs from different kinds of synapses, depending on the neurotransmitters involved, and we thus do not know whether any individual PSD has both the cAMP-activatable and the calmodulin-activatable systems. A partial verification of at least the involvement of cAMP in the modulatory excitatory acetylcholine responses initiated by dopamine has been given by Libet (30).

What we know about these proteins can be summarized as

TABLE III

Summary of Postulated Characteristics of PSDs from Type I and Type II Synapses

Characteristics	Type I	Type II
Function	Excitatory	Inhibitory
Morphology	Thick disk, with large perforation in center; presence of aggregates	Thin disk, lattice-like structure with no large central perforation; little or no aggregates
Enrichment of proteins	Major 51,000 M_r, Protein I, calmodulin	74,000 M_r
Enrichment of calmodulin-dependent phosphorylation	51,000 M_r	58,000 M_r 48,000 M_r
Enrichment of cAMP-dependent phosphorylation	Proteins Ia and Ib	—

follows: 51,000 M_r protein (identity unknown) is phosphorylated by a calmodulin-activatable protein kinase in the PSD (18, 20) and it also binds calmodulin (6, 20); another possible function of calmodulin in the PSD is that it can activate a cAMP-phosphodiesterase (20). The function of proteins Ia and Ib is unknown. While much further work needs to be done to verify this hypothesis for the involvement of the above PSD proteins in the excitatory response, we can state, even now, on the basis of the results of this paper, that the PSD probably has some role in modulating the signal-conduction events occurring at the synapse. However, it should be emphasized that the complete role of the PSD in some signal conduction will only be attained when the PSD is considered as a part of the synapse, as a result of experiments done with systems in which the PSD is still attached to the postsynaptic membrane from which it was isolated.

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