On the identity of the major postsynaptic density protein

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Communicated by Philip Siekert, December 30, 1991

ABSTRACT Increasing evidence suggests that the postsynaptic density (PSD) plays a critical role in synaptic communication and plasticity. The major PSD protein (mPSDp), a calcium/calmodulin-dependent protein kinase, appears to be central to PSD function. The mPSDp has long been considered identical to the α subunit of the soluble calmodulin kinase II (α-CKII). However, mPSDp and α-CKII do differ in solubility and antigenicity, raising the possibility that the two proteins are distinct. To further define the relationship between the two proteins, we purified the mPSDp to homogeneity from adult rat cerebral cortex and compared the proteins. In contrast to α-CKII, the purified mPSDp was insoluble in high concentrations of salt, various detergents, chelators of divalent cations, and the strong denaturant guanidine hydrochloride. The pI value of the mPSDp was 6.2, whereas that of α-CKII was 6.7–7.2. The purified mPSDp bound calmodulin in the presence of Ca²⁺ and was autophosphorylated in a Ca²⁺/calmodulin-dependent manner. Polyclonal antiserum raised against mPSDp (anti-mPSDp) recognized purified mPSDp or mPSDp in synaptic membrane, indicating immunologic specificity among the synaptic proteins. Anti-mPSDp did not recognize α-CKII, whereas anti-α-CKII antibodies reacted only weakly with mPSDp, suggesting that the proteins are distinct but structurally similar. Moreover, sequence analysis of protease V8-digested polypeptides revealed that there was at least an 8-amino acid sequence, MLKVPNIS, that is not present in α-CKII. Finally, HPLC analysis of V8-digested fragments of mPSDp and α-CKII in parallel revealed dissimilar peptide patterns. Thus our observations suggest that mPSDp and α-CKII are similar but not identical. The unique physicochemical and structural properties of the mPSDp may provide insights into molecular mechanisms mediating synaptic plasticity.

Numerous studies suggest that the postsynaptic density (PSD), a proteinaceous disc-shaped structure attached to the postsynaptic membrane of chemical synapses, plays a critical role in synaptic communication and plasticity (for review, see ref. 1). Remarkably, however, only few proteins in the PSD have been characterized biochemically. Cerebral cortical or hippocampal PSDs contain a predominant protein, the major PSD protein (mPSDp), that binds calmodulin (CaM) in the presence of Ca²⁺ (2) and appears to be an autophosphorylating Ca²⁺/CaM-dependent protein kinase (3–5). These unique activities are of critical functional significance since Ca²⁺ influx is a key step in memory formation (6) and since protein phosphorylation is central to signal transduction (7–9). Indeed, by monitoring the mPSDp, we have previously found that impulse activity regulates synaptic molecular architecture in the developing and mature sympathetic superior cervical ganglion (10–12). Moreover, the synaptic molecule mPSDp is similarly regulated in the adult rat hippocampus (12).

Despite its potential functional significance, the identity of the mPSDp and its relationship to the soluble type II Ca²⁺/CaM-dependent protein kinases (CKII)s remain controversial. Soluble CKII s are a group of closely related multifunctional enzymes with broad substrate specificity (13–15). They are large oligomeric proteins composed of 50-kDa (α subunit; α-CKII), 58-kDa (β' subunit), and 60-kDa (β subunit) subunits (16–18). The subunit composition varies from tissue to tissue (16, 19, 20) and with brain region (20, 21). It was reported (3–5) that the mPSDp is identical to the 50-kDa polypeptide (i.e., α-CKII). However, CKII has been localized to the cytosol immunohistochemically (22). In direct contrast, the mPSDp, as part of PSD, is insoluble in high concentrations of salt, various detergents, and the denaturant guanidine hydrochloride (23–28). Moreover, the mPSDp is relatively nonantigenic (K. W. and P. Siekert, unpublished results), in contrast to α-CKII (16, 21). Consequently, the two proteins may not be identical after all.

The present study was designed to further investigate the mPSDp. We have purified the mPSDp to homogeneity and characterized purified mPSDp biochemically and immunologically. Our studies suggest that the mPSDp and α-CKII are structurally related but not identical. These findings may be functionally significant: although α-CKII has been implicated in presynaptic events (29, 30), the mPSDp may be actively involved in postsynaptic function. For example, long-term potentiation and memory may depend on postsynaptic mechanisms (6), involving the mPSDp. The unique physicochemical properties of the mPSDp suggest specific mechanisms through which the protein may mediate these distinct neuronal processes. A preliminary report of this work has appeared (31).

MATERIALS AND METHODS

Materials. CaM was obtained from Sigma. Na⁺(125)I was from Amersham. The other chemicals are of the highest grade from commercial sources.

Isolation of the mPSDp from Cerebral Cortex of Adult Rats. All operations, unless otherwise specified, were performed at 0–4°C. Cerebral cortices from 70 adult rat brains were used for preparation of the mPSDp. Highly purified PSDs were obtained as described (27). The mPSDp was eluted from an SDS gel using 10 vol of 0.1% SDS (1 vol is the volume of the excised gel). The extraction was repeated twice. The mPSDp extracts were combined and concentrated to 5 vol with an Amicon concentrator using the filter membrane PM30. The concentrated solution was treated with 3 vol of acid methanol (adjusted to pH 5.0 with glacial acetic acid) and maintained at −20°C for 2 h. The suspension was centrifuged at 23,000 × g for 15 min. The pellets were resuspended in 5 ml

Abbreviations: PSD, postsynaptic density; mPSDp, major PSD protein; SM, synaptic membrane; CKII, calmodulin kinase II; α-CKII, α subunit of CKII; CaM, calmodulin; IEF, isoelectric focusing.

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of double-distilled water and were then washed sequentially with two 3 vol of acidic methanol and then with three 3 vol of 6 mM Tris-HCl (pH 8.1). The pellet was resuspended in 1 ml of Tris buffer and dialyzed against 4 liters of Tris buffer overnight with one change of buffer. The mPSDp suspension was then stored at ~80°C until used.

PAGE. To assess purity, SDS/PAGE was performed using a 7.5–15% gradient slab gel as described (27). The preparative SDS/PAGE was a slab gel of 10.5% polyacrylamide containing 0.07% N,N'-methylenebisacrylamide. In both cases, the PAGE was at a constant 100 V, as described (27). Isoelectric focusing (IEF) was performed as described by Bio-Rad but using a slab gel instead of a tube gel.

Western Blot Analysis. Synaptic membrane (SM) proteins or the mPSDp on SDS gels were electrophoretically transferred to Immobilon-P as described (32). Anti-mPSDp antiserum–protein binding was performed as described by Towbin et al. (32) with the exception that 125I-labeled protein A was used to detect the initial antibodies bound.

Preparation of Polyclonal Antiserum. The mPSDp in a SDS gel was used to generate polyclonal antibodies. The excised protein bands (~8 µg of mPSDp per band) from the gels were combined and soaked in distilled water for 1 h with three changes. The combined gels were then crushed and homogenized in 1.5 ml of saline by using a syringe and a 20-gauge needle. The mixture was emulsified with 1.5 ml of Freund’s complete adjuvant for two rabbits. The injection (0.2 ml containing ~5 µg of mPSDp) was administered intradermally in the thick skin above the scapula. Subsequently protein bands were emulsified in Freund’s incomplete adjuvant and injected at 3-week intervals. Four weeks after the first injection, a tail bleeding was done from ear veins of the animals. The sera obtained were tested for antibodies against the mPSDp on an immunoblot. Soluble rat brain CKII and polyclonal anti-α-CKII antisera were kindly supplied by A. Czernik and P. Greengard (Rockefeller University). The anti-α-CKII antisemur was prepared by using the peptide corresponding to amino acids 281–302 of soluble α-CKII as immunogen.

Miscellaneous. SM fractions were prepared as described (27) and the protein content was determined by the procedure of Lowry et al. (33), using bovine serum albumin as standard. For peptide mapping, soluble CKII was isolated as described (21) and α-CKII was obtained from CKII by preparative gel electrophoresis used for preparation of the mPSDp. The HPLC analysis of V8-digested mPSDp and α-CKII peptides was performed at the protein sequencing facility of Rockefeller University. Protein sequencing and analysis of amino acid composition of the mPSDp were carried out at the Department of Protein Biochemistry, Columbia University Medical Center. Binding of 125I-labeled CaM to the purified mPSDp was performed as described (2). The Ca2+/CaM-dependent protein kinase activity was measured according to Wu et al. (27).

RESULTS

Purification of the mPSDp. About 0.78 mg of mPSDp was obtained from cerebral cortices collected from 70 adult rat brains, resulting in a 6500-fold purification of the protein based on protein reduction (Table 1). Since both mPSDp and soluble α-CKII (also found in homogenate and synaptosomal fractions) possess similar CaM binding and phosphorylating activities, it was inappropriate to estimate the fold purification of the mPSDp in terms of these activities. Since ~15% of the mPSDp was present in PSDs, based on densitometric scanning of the Coomassie brilliant blue-stained SDS gel (34), there was an 90% recovery of the mPSDp from PSDs (Table 1).

Purity. Apparent Molecular Mass, and pl. On a SDS gel (Fig. 1A) or an IEF gel (Fig. 1B), the preparation exhibited

Cerebral cortices from 70 adult rat brains were used for the preparation of synaptosomes and SMs from which PSDs were isolated. The mPSDp was then purified from PSDs. The fold purification was calculated based on protein reduction.

only one band, at 51 kDa, reflecting apparent homogeneity of the preparation. The pl value calculated from the IEF gel was 6.2, as compared to reported values of 6.7–7.2 (5) and of 6.9 (present study; data not shown) for α-CKII.

Solubility of the Purified mPSDp. mPSDp in the PSD fraction is known to be highly insoluble (23–28). Consequently, the isolated mPSDp was examined for solubility in various solutions, followed by centrifugation and washing of the pellet with 6 mM Tris buffer (pH 8.1). The mPSDp was insoluble in a high concentration of salt (1 M KCl), various detergents (0.5–1.0% Triton X-100, deoxycholate, or N-lauroylsarcosine), chelators of divalent cations (1–10 mM EDTA or EGTA, pH 7.0), or guanidine hydrochloride (4 M, pH 7.0); these characteristics of the purified protein are identical to those reported for the mPSDp localized to the PSD preparation in situ. α-CKII, however, was soluble under the same treatments employed for the mPSDp, suggesting physicochemical differences between the two proteins.

Biochemical Activities of the Purified mPSDp. Binding of CaM. To determine whether the isolated protein retained biological activity, its ability to bind CaM was examined. The preparation failed to bind CaM in the absence of Ca2+ but bound CaM characteristically in the presence of Ca2+ (data not shown). Consequently, the purified protein exhibited activity characteristic of the native mPSDp in the SM or PSD fraction in situ (2).

Ca2+/CaM-dependent protein kinase activity. The purified mPSDp exhibited Ca2+/CaM-dependent autophosphorylation activity (data not shown). Neither Ca2+ nor CaM alone

Table 1. Protein content at each major step of purification of mPSDp from rat cerebral cortex.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein content mg</th>
<th>Fold purification</th>
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<tbody>
<tr>
<td>Homogenate</td>
<td>5089</td>
<td>1</td>
</tr>
<tr>
<td>Synaptosome</td>
<td>1034</td>
<td>5</td>
</tr>
<tr>
<td>SM</td>
<td>199</td>
<td>26</td>
</tr>
<tr>
<td>PSD</td>
<td>5.8</td>
<td>877</td>
</tr>
<tr>
<td>mPSDp</td>
<td>0.78</td>
<td>6524</td>
</tr>
</tbody>
</table>

enhanced phosphorylation, mimicking mPSDp in the SM or PSD fraction (34).

Kinetics of Ca2+/CaM-dependent phosphorylation of the mPSDp. The kinetics of autophosphorylation of the purified mPSDp was examined with respect to protein kinase concentration and time. Autophosphorylation was directly proportional to kinase concentration (1–10 µg, Fig. 2A); phosphate incorporation was linear from 30 sec to 2.0 min and reached a plateau value between 2 and 2.5 min (Fig. 2B). No change in the extent of phosphorylation was found from 2.5 to 5 min of incubation, the longest period tested. The results further suggested that the preparation was pure and free of proteolytic enzymes or phosphatases that may digest the mPSDp or catalyze dephosphorylation.

Amino Acid Analysis of the Purified mPSDp. N-terminal amino acid. Analysis revealed no detectable N-terminal amino acid. Since only one polypeptide moiety was detected in the purified mPSDp (Fig. 1), the N-terminal amino acid was apparently blocked.

Amino acid composition. The amino acid composition indicated that the mPSDp was not typically hydrophobic (Table 2), as reported (35). The polarity index (36), defined as the sum of the residue mole percentages of polar amino acids, was 37.4. The purified protein contained relatively low levels of methionine (0.6%) and tyrosine (3.1%) and relatively high levels of glutamic acid (9.9%), glycine (11.4%), alanine (9.9%), and leucine (9.8%).

Partial amino acid sequence of the mPSDp. To further assess structural relationships between the mPSDp and α-CKII, the mPSDp was digested with V8 protease and two major digested polypeptides at 40 kDa and 8.1 kDa were obtained (data not shown). The 8.1-kDa polypeptide was sequenced, since the N-terminal residue of the other polypeptide was blocked. The 8.1-kDa polypeptide contained an 8-amino acid sequence, MLKVPNIS, that is not present in α-CKII (37). The findings suggested that the mPSDp and α-CKII are distinct.

HPLC Analysis of V8-Digested mPSDp and α-CKII Peptides. To further compare mPSDp and α-CKII directly, the two proteins were subjected to SDS/PAGE and were transferred to Immobilon-P. The proteins transferred were stained with ponceau S, the bands containing the specific protein were digested with V8 protease, and the resulting peptides were HPLC analyzed. Analysis revealed that V8 cleaved mPSDp and α-CKII similarly but nonidentically; at least 12 peptides were generated from each protein, but these peptides displayed different patterns (Fig. 3). These results further suggest that the two proteins are similar but distinct.

Specificity of Anti-mPSDp Antiserum. The purified mPSDp was injected into two rabbits for the production of polyclonal antibodies. Immunoblot analysis indicated that the anti-mPSDp antiserum recognized a single band at 51 kDa using either purified mPSDp (Fig. 4A) or highly purified SM

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol %</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Asp</td>
<td>6.9</td>
<td>13.0</td>
</tr>
<tr>
<td>Glu</td>
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<td>19.0</td>
</tr>
<tr>
<td>Ser</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>11.0</td>
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<tr>
<td>Cys</td>
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</tr>
<tr>
<td>Ile*</td>
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</tr>
<tr>
<td>Leu</td>
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</tr>
<tr>
<td>Lys</td>
<td>7.2</td>
<td>13.9</td>
</tr>
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mPSDp was purified from cerebral cortex of adult rat brain and was assayed for amino acid composition. —. Not determined.

*Isoleucine was used to calculate the molar ratio of the amino acids.

fractons (Fig. 4 B and C). Consequently, the polyclonal antibodies appeared to be highly specific for the mPSDp. However, the antibodies titer was apparently low, since no bands were detectable at a dilution of antisera >1:5 (Fig. 4).

The contention that the mPSDp itself is a Ca2+/CaM-dependent protein kinase was verified by showing that the polyclonal anti-mPSDp antiserum inhibited the Ca2+/CaM-dependent protein kinase activity (data not shown).

Immunochenical Differences Between the mPSDp and α-CKII. To further compare the structural relationship be-

![Fig. 2. Properties of Ca2+/CaM-dependent protein kinase activity found in the mPSDp. Purified mPSDp (5 µg) was used for each experiment. (A) Phosphate incorporation is a function of the amount of mPSDp present. (B) Phosphate incorporation as a function of incubation time. Ca2+, 0.2 mM; CaM, 3 µg.](image)

![Fig. 3. HPLC analysis of the V8-digested peptides from mPSDp and α-CKII. mPSDp (10 µg) and α-CKII (10 µg) were transferred to Immobilon-P and the proteins were stained with ponceau S. The bands containing mPSDp and α-CKII were digested with V8 protease and the resulting peptides were analyzed by HPLC. Arrows indicate the peptides not shared by mPSDp and α-CKII, with dotted lines connecting the corresponding peptides in the same retention time. mAU, milliabsorption unit(s). (Upper) mPSDp. (Lower) α-CKII.](image)

Table 2. Amino acid composition of the mPSDp
between mPSDp and α-CKII, reactions with anti-mPSDp antiserum or anti-α-CKII antiserum were examined. At an equimolar concentration of antigen, anti-mPSDp antiserum recognized mPSDp but not α-CKII (data not shown), indicating physicochemical differences between the two antigens. Moreover, anti-α-CKII antiserum reacted with apparently 10-fold greater avidity with α-CKII than with mPSDp (Fig. 5). Similar results were obtained using α-CKII pre-treated as mPSDp. Consequently, though immunologically distinct, the two antigens may share structural characteristics.

**DISCUSSION**

We have described the purification to homogeneity of the mPSDp from cerebral cortex of the adult rat. The purified protein was characterized biochemically and immunologically and was compared to α-CKII.

**Physicochemical Characterization. Biological activities of the purified mPSDp.** The purified kinase had the same apparent molecular mass (51 kDa) and CaM-binding and Ca\(^{2+}\)/CaM-dependent phosphorylation activities as the mPSDp within the SM or PSD in situ. Thus, these unique biologic activities of the mPSDp are apparently intrinsic and independent of interactions with other components of the SM or PSD. Consequently, the mPSDp itself may mediate the Ca\(^{2+}\)/CaM-dependent phosphorylation that appears to be pivotal in synaptic communication and long-term synaptic change (7, 8, 38). For example, Ca\(^{2+}\)/CaM-dependent phosphorylation of the mPSDp may alter the molecular conformation leading to changes in PSD shape and synaptic spine morphology with altered conductance (1, 7).

**pI.** The pI value of the purified mPSDp was 6.2, in contrast to a value of 6.9 from the present study and the reported value of 6.7–7.2 for the soluble α-CKII (5); consequently, the two proteins were physicochemically different. Thus, at physiological pH (7.4), the mPSDp is slightly more positively charged than α-CKII, which bears almost no net charge at this pH. The differences may be functionally significant. For example, under physiological conditions, the mPSDp and α-CKII may bind substrates with different affinities resulting in different reaction rates with consequentially different synaptic effects.

**Solubility.** The isolated mPSDp was insoluble in high concentrations of salt, divalent cation chelators, various detergents, and the strong denaturing reagent guanidine hydrochloride, mimicking insolubility of the mPSDp within the PSD. Consequently, insolubility of the mPSDp appears to be an intrinsic property of the molecule and not dependent on interactions with other components of the PSD. Insolubility of the mPSDp contrasts sharply with the properties of α-CKII, which is essentially localized in the cytosol (22) and appears to be soluble when treated as mPSDp. Indeed, this difference in solubilities suggests functional distinctions between the proteins. For instance, the insolubility itself may result in a low turnover of the mPSDp, permitting relatively long-term stable structural synaptic change.

**Partial amino acid sequence and peptide maps.** The 8-amino acid peptide MLKVPNIS of the mPSDp is not present in soluble α-CKII. Examination of the protein database (Protein Identification Resources; search date, March 22, 1990) revealed that the sequence LKVPN is also present in neumaminidase (39). However, the amino acid composition of the mPSDp is markedly different from that of neuraminidase, excluding contamination by the latter. Although the complete amino acid sequence of the mPSDp remains to be determined, our observations suggest that the mPSDp and α-CKII exhibit sequence differences. Moreover, parallel HPLC analyses of the V8-digested peptides from mPSDp and α-CKII revealed distinct peptide mapping, confirming that the two proteins are nonidentical.

**Immunological Characterization.** Polyclonal anti-mPSDp antiserum specifically recognized the purified mPSDp and the mPSDp as a component of the total SM fraction. Moreover, the antiserum specifically inhibited Ca\(^{2+}\)/CaM-dependent protein kinase activity of the mPSDp, confirming that the purified mPSDp was a protein kinase. These results suggest that the antiserum was specific for the mPSDp and that the purified protein was catalytically active. In contrast, the anti-mPSDp antiserum did not recognize the soluble α-CKII, as reported (35). The results support the contention

*Fig. 5.* Reaction of anti-α-CKII antibodies with CKII and mPSDp. mPSDp (5.00 μg) and CKII (6.67 μg) were subjected to SDS/PAGE, and proteins in the gel were transferred to nitrocellulose for immunoblot analysis with anti-α-CKII antibodies and autoradiography. Lanes: A, CKII; B, mPSDp. Anti-α-CKII antibodies were used at a 1:2000 dilution.

*Fig. 4.* (A) Immunoblot analysis of anti-mPSDp antibodies. Purified mPSDp (5 μg) was subjected to SDS/PAGE and was transferred to nitrocellulose for immunoblot and autoradiographic analyses. (B and C) Reaction of anti-mPSDp antiserum with antigens in the highly purified SM preparation. SM protein (40 μg) was fractionated on a SDS gel and the separated proteins were transferred to nitrocellulose for immunoblot analysis. (B) Coomassie brilliant blue-stained profile of the SM proteins. (C) Autoradiogram of the immunoblotted band. Dilation factors of anti-mPSDp antiserum are lane labels.
that mPSDp and α-CKII are not identical. Conversely, antiserum raised against α-CKII had at least a 10-fold higher avidity against α-CKII than mPSDp. Thus these findings suggest that the mPSDp and α-CKII are structurally similar but not identical.

In summary, the conclusion of the earlier papers (3–5) concerning the identity of the mPSDp and α-CKII was based chiefly on the similar molecular masses, CaM binding, and Ca\(^{2+}\)/CaM-dependent phosphorylating activities, pl values, tryptic/chymotryptic maps determined by PAGE, and immunological characterization using anti-α-CKII antiserum. However, our present work revealed that the two proteins have different pl values. Further, anti-mPSDp antiserum did not react with α-CKII, and anti-α-CKII antiserum reacted strongly with α-CKII but only weakly with mPSDp. Most importantly, the unique 8-amino acid peptide of the mPSDp was absent in the α-CKII, and the two proteins yielded dissimilar V8 peptide maps, determined by HPLC. Consequently, we conclude that mPSDp and α-CKII are similar but nonidentical.

Results of the present studies support the existence of distinct brain Ca\(^{2+}\)/CaM-dependent protein kinases. To define the physiological actions of Ca\(^{2+}\) that are mediated by protein phosphorylation, it will be necessary to characterize each kinase. The exact physiological role of the mPSDp remains to be defined. Nevertheless, available evidence suggests that the soluble CKII regulates presynaptic functions (29,30), whereas the mPSDp may play a critical role in postsynaptic mechanisms. The high concentration of the mPSDp in PSDs, its physicochemical characteristics, and its Ca\(^{2+}\)/CaM-dependent phosphorylating activity may confer unique responsiveness to Ca\(^{2+}\) flux and transmitters.

We thank Betty Wheeler for excellent technical assistance and Drs. Andrew Czernik and Paul Greengard (Rockefeller University) for generous supplies of CKII and anti-α-CKII antiserum. This work was supported by National Institutes of Health Grants NS 10259 and HD 23315, a March of Dimes National Foundation Grant, and a Bristol-Myers Squibb Co. Unrestricted Neurosciences Research Grant. I.B.B. is the recipient of a McKnight Research Project Award.