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Evidence that the major postsynaptic density protein is a component of a Ca^{2+}/calmodulin-dependent protein kinase

(synaptic junction/phosphorylation/peptide mapping/immunoblot)

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ABSTRACT **Polypeptides of M_r 50,000 and 60,000 in isolated synaptic junctions have been compared to polypeptides of corresponding molecular weight in Ca^{2+}/calmodulin-dependent protein kinase II. The polypeptides of corresponding molecular weight from the two preparations were shown by several criteria to be indistinguishable. These criteria included ^{125}I-labeled tryptic/chymotryptic peptide patterns, ^{32}P-labeled proteolytic peptide maps, and crossreactivity on immunoblots using polyclonal and monoclonal antibodies. Furthermore, studies examining the phosphorylation of substrate proteins, by the endogenous synaptic junction kinase and by Ca^{2+}/calmodulin-dependent protein kinase II, indicated that the two enzymes have similar substrate specificities. Since the M_r 50,000 polypeptide present in synaptic junctions is known to be the major postsynaptic density protein, the present results indicate that the major postsynaptic density protein is a component of Ca^{2+}/calmodulin-dependent protein kinase II.**

A number of Ca^{2+}/calmodulin-dependent protein kinases have recently been identified in neuronal tissue (for review, see ref. 1). One of these is a kinase purified from rat brain (2) that phosphorylates synapsin I, a synaptic vesicle-associated neuronal protein (3, 4). This kinase, designated Ca^{2+}/calmodulin-dependent protein kinase II (calmodulin kinase II), has recently been purified to near homogeneity (5, 6) and exhibits a major M_r 50,000 polypeptide and a less prominent M_r 60,000 polypeptide when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. These M_r 50,000 and 60,000 polypeptides coelute with the peak of kinase activity through a variety of purification procedures, are themselves phosphorylated in a Ca^{2+}/calmodulin-dependent manner, and are calmodulin-binding proteins (5, 6).

Grab *et al.* (7) found a calmodulin-sensitive protein kinase in a postsynaptic density (PSD) preparation isolated from canine cerebral cortex. An endogenous M_r 50,000 polypeptide is both a substrate for this kinase (7) and a calmodulin-binding protein (8). This M_r 50,000 polypeptide is the most abundant PSD protein and has been designated the major PSD protein (mPSDp) (9–11). This PSD preparation also contains a substrate protein (7) and a calmodulin-binding protein (8) in the M_r 60,000 range. Substrate proteins (12) and calmodulin-binding proteins (13, 14) of M_r 50,000 and 60,000 have also been observed in PSD and synaptic junction (SJ) preparations isolated from rat forebrain. The SJ M_r 50,000 polypeptide is identical to the mPSDp (11, 14). Therefore, either SJ or PSD preparations can be used as a source for the mPSDp. The similarities between the M_r 50,000 and 60,000 polypeptides in calmodulin kinase II and those of corresponding molecular weight in SJ preparations prompted us to examine their possible relationship. A preliminary account of this work has been reported (15).

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METHODS

Subcellular Fractionation. Whole forebrains were used to isolate SJ fractions by the iodonitrotetrazolium violet/Triton X-100 method as described (11). Care was taken to remove residual Triton X-100 from the centrifuge tube walls by washing a minimum of five times with 3 ml of 2 mM Hepes (pH 7.2) prior to final harvesting of SJ pellets. Pellets were then suspended, by homogenization in 2 mM Hepes, to a concentration of 1–3 mg/ml and were stored at −80°C. Protein concentrations were determined as described (11).

Standard Phosphorylation Assays. Assays for calmodulin kinase II and SJ kinase activity and the *in vitro* phosphorylation of endogenous calmodulin kinase II and SJ polypeptides were carried out by one of two procedures. The first procedure (assay 1) was carried out as described (2) except that the reaction mixture contained 3 μg of calmodulin (purified as described in ref. 16) and 10 μM [γ-^{32}P]ATP, and the reaction was carried out for 10 sec. The second procedure (assay 2) was done in a reaction mixture (final vol, 100 μl) containing 10 mM Hepes, pH 7.5/5 mM $MgCl_2$/0.5 mM dithiothreitol/0.5 mM EGTA/0.7 mM $CaCl_2$/15 μM [γ-^{32}P]ATP (3.5 μCi per reaction; 1 Ci = 37 GBq)/2 μg of calmodulin (Calbiochem) and various amounts of SJ protein or calmodulin kinase II. Some reaction mixtures contained 1–10 μg of purified synapsin I (purified as described in ref. 2). Phosphorylation was initiated by the addition of [γ-^{32}P]ATP and incubations were at 30°C for 1 min. Reactions were terminated by the addition of $NaDodSO_4$ buffer and reaction mixtures were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described (17). Molecular weight standards were as follows: phosphorylase b, M_r 94,000; bovine serum albumin, M_r 68,000; ovalbumin, M_r 43,000; chymotrypsinogen, M_r 25,000; myoglobin, M_r 17,000.

Purification of Calmodulin Kinase II. Calmodulin kinase II was purified from rat brain by modification of the procedure described by Kennedy *et al.* (2). Briefly, the cytosol and extracted particulate fractions from rat forebrain homogenates were pooled and subjected to DEAE-cellulose chromatography, 35% ammonium sulfate precipitation, gel filtration on Sephacryl S-400, and calmodulin-Sepharose affinity chromatography. The final kinase preparation exhibited a specific activity of 4 μmol mg^{-1} min^{-1} using synapsin I as substrate.

Peptide Mapping. Two-dimensional peptide mapping of proteins radioiodinated with ^{125}I in gel slices was carried out as described (11), except that proteins were digested with a mixture of equal amounts (50 μg/ml) of trypsin and chymotrypsin. Electrophoresis was from left to right in the first dimension and chromatography was from bottom to top in the second dimension (see Figs. 2, 3, and 6). Five separate peptide mapping experiments were done. One-dimensional ^{32}P-

Abbreviations: SJ, synaptic junction; PSD, postsynaptic density; mPSDp, major PSD protein.

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labeled proteolytic peptide mapping was carried out as described (2).

Transfer of Proteins from $NaDodSO_4$/Polyacrylamide Gels to Nitrocellulose and Detection of Antigens by Immunoblotting. ^{32}P-labeled SJ proteins (15 μg) and calmodulin kinase II proteins (2.5 μg) were separated on $NaDodSO_4$/polyacrylamide gels (2) and electrophoretically transferred to nitrocellulose by modification (18) of the method of Towbin *et al.* (19). Immunoblotting was as described (18) using a 1:300 dilution of mouse monoclonal antibody C42.1 and a 1:400 dilution of goat anti-mouse IgG $F(ab')^2$ fragment conjugated to alkaline phosphatase.

Production of Monoclonal Antibodies. Monoclonal antibodies against calmodulin kinase II M_r 50,000 and 60,000 polypeptides were produced by modification of the method of Köhler and Milstein (20). Hybridomas secreting antibodies to the M_r 50,000 and 60,000 polypeptides were selected by screening culture supernatants for their ability to immunoprecipitate ^{125}I-labeled polypeptides.

RESULTS

The protein staining pattern and autoradiogram of endogenously phosphorylated polypeptides present in the highly purified preparation of calmodulin kinase II are shown in Fig. 1. This enzyme preparation exhibited a major protein staining band of M_r 50,000 and less prominent protein staining band of M_r 60,000. The M_r 50,000 and 60,000 polypeptides in calmodulin kinase II were extensively phosphorylated in the presence of Ca^{2+} and calmodulin (Fig. 1). Isolated SJ preparations contain polypeptides of M_r 50,000 and 60,000 that were also phosphorylated in a Ca^{2+}/calmodulin-dependent manner and comigrated with the calmodulin kinase II polypeptides. Calmodulin kinase II and SJ preparations also contained a minor protein staining band of M_r 58,000 that was phosphorylated in a Ca^{2+}/calmodulin-dependent manner.

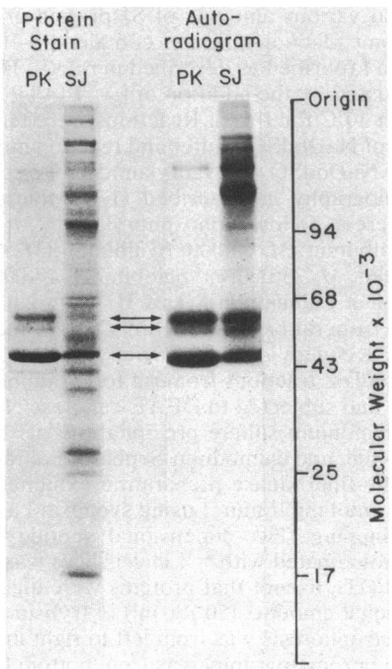

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis showing protein staining pattern and autoradiogram of endogenously phosphorylated polypeptides present in calmodulin kinase II (PK) and in a SJ preparation. PK (4 μg) and SJ (30 μg) were each phosphorylated using assay 1. One microgram of phosphorylated PK was added to 9 μg of unlabeled PK prior to electrophoresis. Arrows point to the M_r 60,000, 58,000, and 50,000 polypeptides.

Comparison of SJ Polypeptides with Calmodulin Kinase II Polypeptides of Corresponding Molecular Weight. *^{125}I-labeled tryptic/chymotryptic peptide patterns.* Evidence that the M_r 50,000, 58,000, and 60,000 polypeptides in SJ preparations are highly related, if not identical, to the polypeptides of corresponding molecular weight in calmodulin kinase II was obtained by two-dimensional patterns of ^{125}I-labeled peptides. About 40 ^{125}I-labeled peptides were obtained in each pattern. Fig. 2 shows that the patterns of the M_r 50,000 polypeptides from calmodulin kinase II (Fig. 2*A*) and SJ preparations (Fig. 2*C*) were virtually identical. Furthermore, the pattern of a mixture of equal amounts (cpm) of the two digests (Fig. 2*B*) shows that the two-dimensional positions of their prominent peptides were indistinguishable.

Patterns of the M_r 60,000 polypeptides from calmodulin kinase II and SJ preparations are shown in Fig. 3*A* and *C*. There were $\approx$40 ^{125}I-labeled peptides in each pattern, of which 32 were common to both preparations. The pattern of a mixture of equal amounts (cpm) of the two digests (Fig. 3*B*) shows that the M_r 60,000 polypeptides from the two sources are highly related. In addition, the ^{125}I-labeled peptide patterns of the M_r 58,000 polypeptides from calmodulin kinase II and SJ preparations indicate that these polypeptides are also highly related (data not shown).

^{32}P-labeled Staphylococcus aureus *V8 protease peptide maps.* When either calmodulin kinase II or a SJ preparation

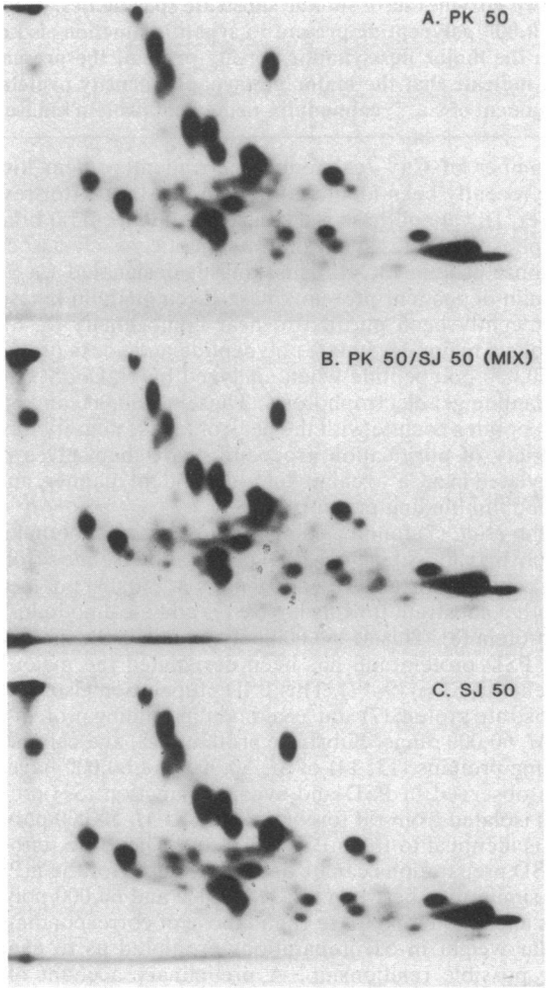

FIG. 2. Two-dimensional ^{125}I-labeled peptide patterns of (*A*) M_r 50,000 polypeptide from calmodulin kinase II (PK 50), (*B*) a mixture of equal amounts (cpm) of M_r 50,000 polypeptides from calmodulin kinase II and SJ preparations, and (*C*) M_r 50,000 polypeptide from a SJ preparation (SJ 50). Each sample contained 5×10^5 cpm.

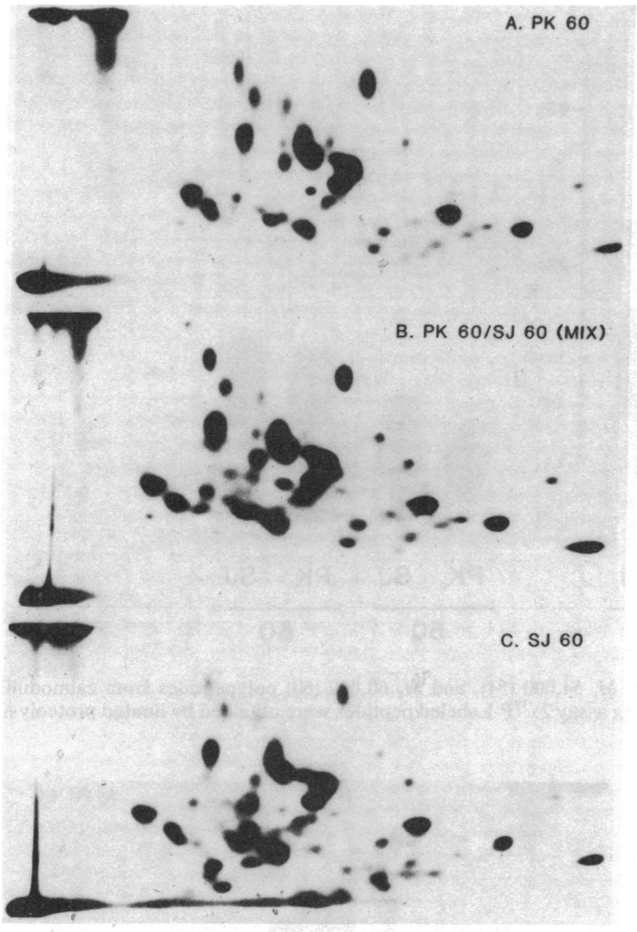

FIG. 3. Two-dimensional ^{125}I-labeled peptide patterns of (*A*) M_r 60,000 polypeptide from calmodulin kinase II (PK 60), (*B*) a mixture of equal amounts (cpm) of M_r 60,000 polypeptides from calmodulin kinase II and SJ preparations, and (*C*) M_r 60,000 polypeptide from a SJ preparation (SJ 60). The SJ M_r 60,000 polypeptide was electrophoretically purified on a second NaDodSO$_4$/polyacrylamide gel prior to iodination. Each sample contained 5×10^5 cpm.

was incubated *in vitro* for short periods (10–30 sec) in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin, endogenous phosphorylation of M_r 50,000, 58,000, and 60,000 polypeptides was observed (see Fig. 1). After longer periods of phosphorylation, an additional M_r 54,000 radioactive band was observed in both calmodulin kinase II and SJ preparations (data not shown). In addition, when either type of preparation was phosphorylated for more than 30 sec, the M_r 50,000 protein staining band was transformed into a doublet of M_r 50,000 and 54,000 upon NaDodSO$_4$/polyacrylamide gel electrophoresis (data not shown). One-dimensional proteolytic peptide maps of the M_r 50,000, 54,000, and 60,000 ^{32}P-labeled polypeptides from calmodulin kinase II and SJ preparations are shown in Fig. 4. The ^{32}P-labeled peptide maps of the SJ phosphoproteins were indistinguishable from those of corresponding molecular weight in calmodulin kinase II, when either 0.75 or 15 μg of *S. aureus* V8 protease was used. Similarly, the M_r 58,000 polypeptides from the two sources yielded indistinguishable ^{32}P-labeled peptide maps (data not shown).

Immunoblots. Monoclonal antibodies and polyclonal antisera were obtained against purified mPSDp (unpublished results) and against calmodulin kinase II (unpublished results; ref. 5). The immunoreactivity of one anti-calmodulin kinase II antibody, as shown by the immunoblot technique, is shown in Fig. 5. This monoclonal antibody (C42.1), as well as all other antibodies examined to date, crossreacted with the M_r 50,000, 58,000, and 60,000 phosphopeptides in both SJ and calmodulin kinase II preparations. These results provide additional evidence for a close relationship between SJ and kinase polypeptides of corresponding molecular weight. The results also indicate that the M_r 50,000, 58,000, and 60,000 polypeptides from calmodulin kinase II and from SJ preparations are immunologically similar.

Comparison of the M_r 60,000 and 50,000 Polypeptides. The ^{125}I-labeled peptide patterns of the calmodulin kinase II M_r 60,000 and 50,000 polypeptides showed that the two polypeptides are similar but not identical (Fig. 6). Twenty-six peptides were common to both the M_r 60,000 and 50,000 polypeptides; five and three ^{125}I-labeled peptides were greatly enriched in the maps of the M_r 60,000 and 50,000 polypeptides, respectively. Similar results were obtained for the M_r 60,000 and 50,000 polypeptides from the SJ preparation (data not shown). In contrast to the similarities between the ^{125}I-labeled peptide patterns of the M_r 50,000 and 60,000 polypeptides (Fig. 6), the ^{32}P-labeled peptide maps were quite different (Fig. 4). This was true with either calmodulin kinase II or SJ preparations.

Proteolytic phosphopeptide mapping showed that the M_r 50,000 and 54,000 phosphoproteins from calmodulin kinase II and SJ preparations contained three major phosphopeptides in common (M_r ≈28,000, ≈14,000, and ≈11,000; Fig. 4). These results are compatible with the interpretation (see above) that the M_r 54,000 polypeptide is a highly phosphorylated form of the M_r 50,000 polypeptide.

For both SJ and calmodulin kinase II preparations, the ^{125}I-labeled peptide map of the M_r 58,000 polypeptide was compared with, and found to be virtually identical to, that of the M_r 60,000 polypeptide in the same preparation (data not shown). These observations, together with previous ^{32}P-labeled peptide mapping results (2), indicate that the two polypeptides are related. The M_r 58,000 component may be derived from the M_r 60,000 polypeptide by proteolysis.

Comparison of Substrate Protein Phosphorylation by Calmodulin Kinase II and SJ Kinase. To compare the substrate specificities of calmodulin kinase II and SJ kinase, phosphorylation of endogenous SJ proteins (50 μg) was examined in the absence or presence of purified calmodulin kinase II (0.5–1.5 μg) by using assay 2. Under conditions in which phosphorylation rates were linear, calmodulin kinase II increased the state of phosphorylation of only those SJ proteins that were phosphorylated by the endogenous SJ kinase, with the exception of the M_r 60,000 and 50,000 phosphoproteins. Quantitatively, SJ phosphoproteins of M_r 240,000, 200,000, 170,000, and 140,000 incorporated 51%, 69%, 46%, and 77% more ^{32}P, respectively, when SJ fractions were incubated in the presence of exogenous kinase than when incubated alone (n = 4). Under the same conditions, the phosphorylation of the M_r 60,000 and 50,000 phosphoproteins was not significantly increased above the values obtained when SJ preparations were incubated alone (n = 5).

Additional experiments showed that the SJ kinase phosphorylated exogenous synapsin I in a Ca^{2+}/calmodulin-dependent manner. During a 1-min standard phosphorylation reaction using assay 2, with saturating synapsin I (0.1 mg/ml), the SJ kinase phosphorylated synapsin I to the extent of 0.48 mol/mol. Proteolytic phosphopeptide mapping of ^{32}P-labeled synapsin I using *S. aureus* V8 protease showed that the SJ kinase phosphorylated the same M_r 30,000 region of synapsin I that is phosphorylated by calmodulin kinase II (2, 21) (data not shown).

DISCUSSION

The results presented here show that the M_r 50,000 polypeptides in SJ preparations and calmodulin kinase II are virtually identical. Similarly, the M_r 60,000 polypeptides in the two preparations are highly related if not identical. The relation-

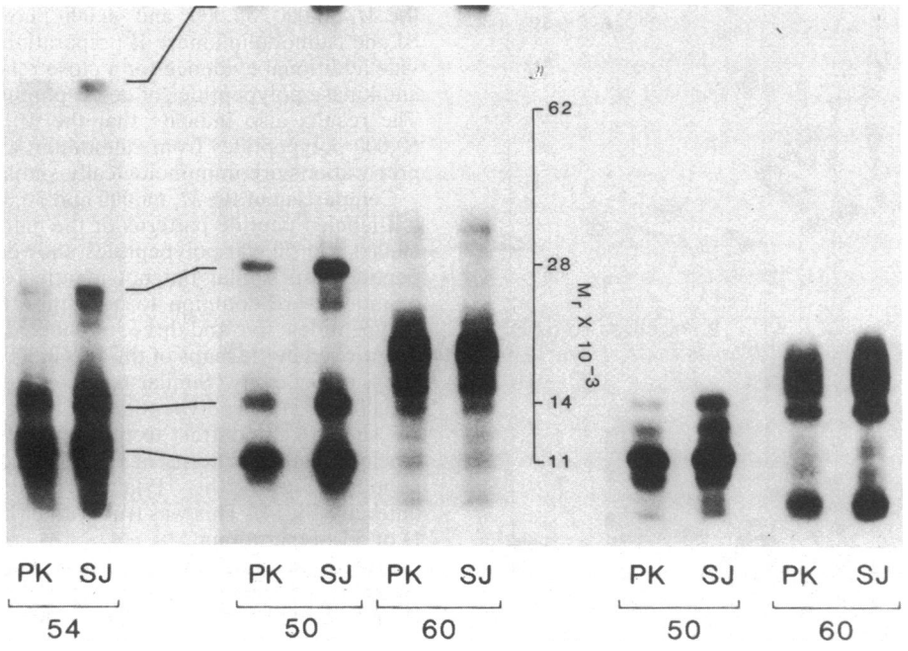

FIG. 4. One-dimensional ^{32}P-labeled peptide maps of M_r 50,000 (50), M_r 54,000 (54), and M_r 60,000 (60) polypeptides from calmodulin kinase II (PK) and SJ preparations. Polypeptides were phosphorylated using assay 2. ^{32}P-Labeled peptides were obtained by limited proteolysis using either 0.75 μg (*Left*) or 15 μg (*Right*) of *S. aureus* V8 protease.

ship between the M_r 50,000 and 60,000 polypeptides remains to be determined. Results from the ^{125}I-labeled peptide patterns and immunoblots indicate that these polypeptides are structurally similar but not identical. In contrast to the ^{125}I-labeled peptide mapping results, the ^{32}P-labeled peptide maps of the two phosphoproteins were quite dissimilar (Fig. 4; ref. 2). The basis for these different peptide mapping results is unclear.

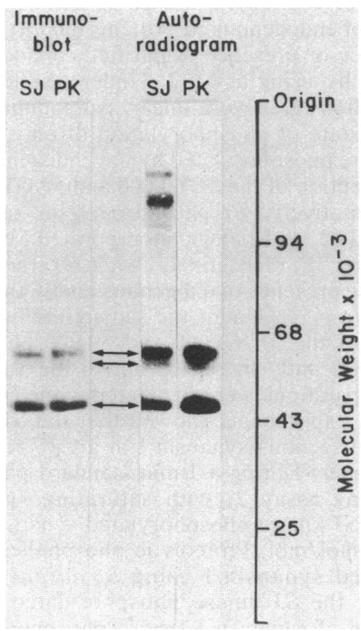

FIG. 5. Immunoblot and autoradiogram of calmodulin kinase II (PK) and SJ preparations after transfer to nitrocellulose. PK (2.5 μg) and SJ (15 μg) preparations were phosphorylated using assay 1, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Immunoblotting was carried out using monoclonal antibody C42.1. Arrows point to the M_r 60,000, 58,000, and 50,000 polypeptides.

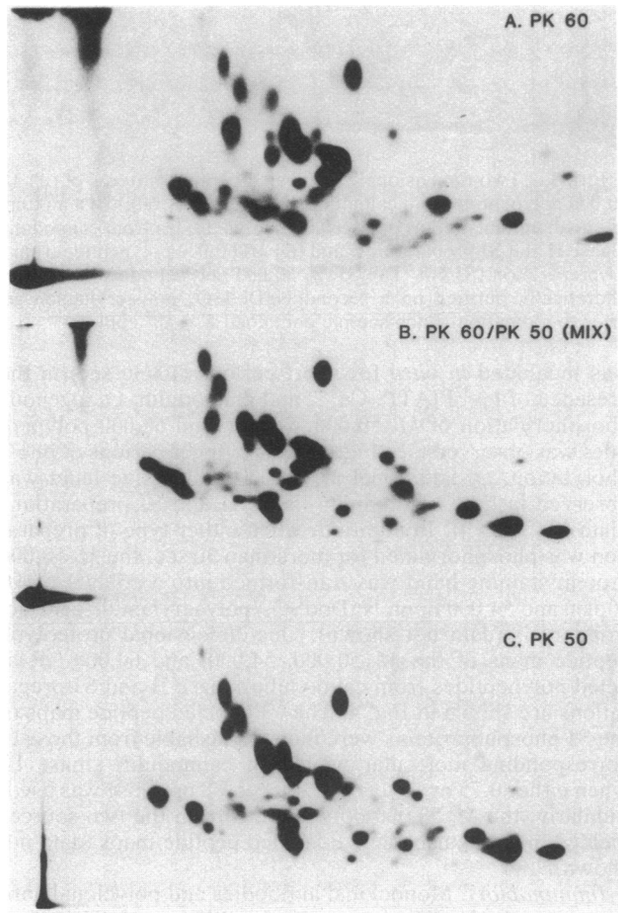

FIG. 6. Two-dimensional ^{125}I-labeled peptide patterns of (*A*) M_r 60,000 polypeptide from calmodulin kinase II (PK 60), (*B*) a mixture of equal amounts (cpm) of M_r 60,000 and 50,000 polypeptides from calmodulin kinase II, and (*C*) M_r 50,000 polypeptide from calmodulin kinase II (PK 50). Each sample contained 5×10^5 cpm.

Previous results on the distribution of the M_r 50,000 mPSDp (11) in neural and non-neural tissue indicated that this protein was present only in brain and only in those subcellular fractions containing identifiable synaptic junctions or postsynaptic densities. In light of the present findings, together with the observation that the mPSDp binds calmodulin (8, 13, 14) and the demonstration that approximately half of the calmodulin kinase II activity in rat brain homogenates is soluble (2, 21), the distribution of the M_r 50,000 mPSDp in subcellular fractions from rat brain has been reexamined. By using the ^{125}I-labeled calmodulin gel overlay method (8) we have found the mPSDp to be present in cytosolic fractions but in $\approx$1/10th the amount (per mg total protein) as that in SJ preparations (data not shown). Previous conclusions (11) were based on ^{125}I-labeled peptide patterns of the M_r 50,000 region of NaDodSO$_4$/polyacrylamide gels and can be explained by the presence in cytosolic fractions of a prominent polypeptide that comigrates with the mPSDp but does not bind calmodulin. Thus, detection of small amounts of the mPSDp in cytosolic fractions was obscured by the presence of this unrelated M_r 50,000 protein.

Previously, it was found that much of the particulate-associated calmodulin kinase II activity was easily extracted by dilution of the particulate material into low ionic strength buffers (2). In the present experiments it was found that neither calmodulin kinase II activity nor the M_r 50,000 mPSDp present in SJ preparations was solubilized either by the same or by other extraction procedures (data not shown). Therefore, calmodulin kinase II appears to exist in at least three subcellular compartments: soluble, loosely associated with particulate fractions, and tightly bound to synaptic junctions. The results presented here support the possibility that calmodulin kinase II, of which the mPSDp is a component, may play an important role *inter alia* in postsynaptic neuronal function.

Note Added in Proof. After this paper was communicated, an article by Kennedy *et al.* (22) appeared showing similar results.

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