

Calmodulin-dependent Protein Phosphatase: Immunocytochemical Localization in Chick Retina

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ABSTRACT Calmodulin-dependent protein phosphatase, previously called CaM-BP80 or calcineurin, is present in high concentrations in the central nervous system. The level of the phosphatase has been shown by radioimmunoassay to increase during development in the retinas of embryonic and hatchling chicks (Tallant, E. A., and W. Y. Cheung, 1983, *Biochemistry*, 22:3630–3635). The aims of this study are to immunocytochemically localize the phosphatase in developing and mature retinas and to determine if the phosphatase is present in fractions of retinal synaptic membranes and synaptic junctions. Vibratome slices of fixed chick retina and Western blots of detergent-solubilized retinal fractions are both treated sequentially with rabbit primary antisera and goat anti-rabbit Fab fragments conjugated to peroxidase, and then reacted with hydrogen peroxide and diaminobenzidine. The tissue slices are further processed for electron microscopy. This paper demonstrates the presence of peroxidase reaction product in the retina just before synapse formation. In the outer plexiform layer the product is confined to photoreceptor synaptic terminals, whereas in the inner plexiform layer it is present in synaptic terminals of bipolar cells and in dendrites of ganglion cells. In this latter site the product is present postsynaptically at bipolar and amacrine synapses. The phosphatase is detected in Western blots of both synaptic plasma membrane and synaptic junction fractions.

Calmodulin-dependent phosphorylation and dephosphorylation of protein are likely to be important regulatory events in cellular activity (1, 2, 24). Calmodulin-dependent protein phosphatase has been shown to dephosphorylate a number of proteins (15, 25, 27, 30). The enzyme is a heterodimer with a subunit A (M_r 60,000), which harbors the catalytic site (34), and a subunit B (M_r 16,000), which contains four calcium binding sites (21). It is present in high concentrations in the brain (33) and is therefore of special interest in light of known calcium influences on neural activities. The phosphatase, identical to calcineurin (29, 38) and previously called CaM-BP80 (32), has been localized by immunocytochemistry to dendritic processes in the rat brain where it is primarily associated with postsynaptic densities (35–37). By radioimmunoassay, the level of protein phosphatase has been found to increase in the brain and retina during a period of embryogenesis (29) that may correlate with the period when large numbers of synapses are forming.

Synaptogenesis in the outer plexiform layer of the chick retina consists of a sequence of events in which photoreceptors form two morphologically distinct types of junction with two major cell classes, the horizontal and the flat bipolar. Photoreceptor junctions with horizontal cells are called ribbon junctions due to the presence of a lamellar-like presynaptic density. These ribbon junctions can be readily distinguished from photoreceptor basal junctions with flat bipolar cells which are located more distally and which contain paramembranous densities at photoreceptor presynaptic sites. The ribbon junctions develop between embryonic days 15 and 18, before the formation of basal junctions, and presynaptic structures develop before postsynaptic structures (26).

The aims of this study are to use immunocytochemistry to determine if the appearance of the enzyme correlates with synaptogenesis in the chick embryo, to localize the enzyme in the hatched chick retina, and to determine if the enzyme is present in fractions of retinal synaptic membranes.

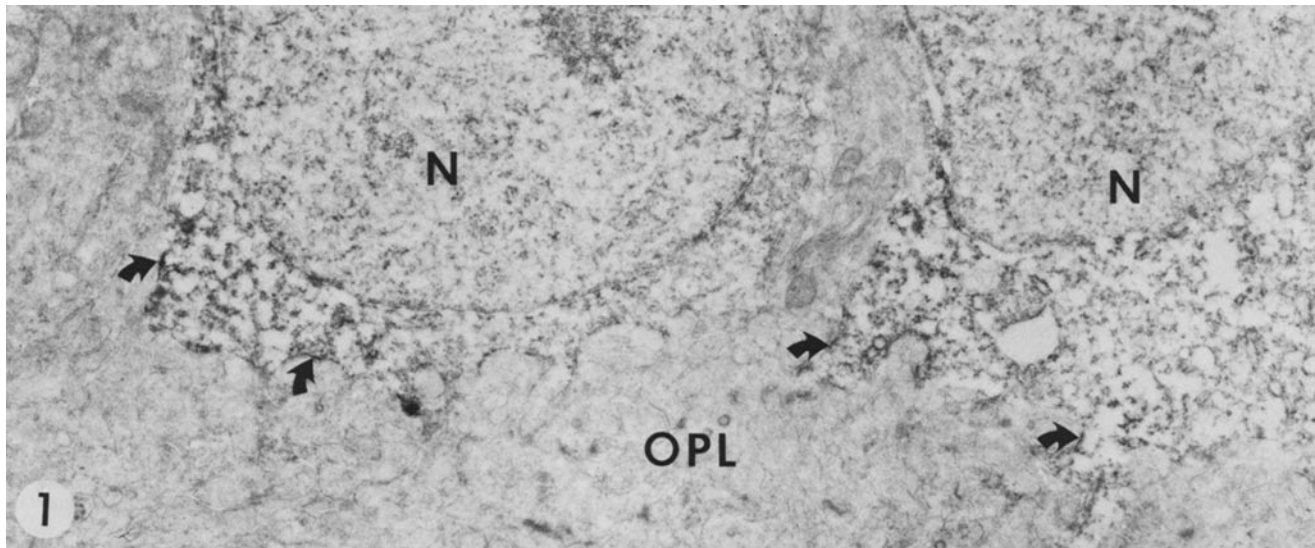


FIGURE 1 13-d unstained, embryonic retina. OPL, outer plexiform layer. N, photoreceptor nucleus. Photoreceptor pedicles before the formation of synapses contain reaction product which is found in patches along the plasmalemma (arrows) and within the cytoplasm. $\times 22,750$.

Electron microscopy (EM)¹ is used to show that the enzyme is present in putative synaptic locations at embryonic times. Using this technique, we found the enzyme to be associated with the structural elements of synapses as they appear during development. Using a second technique, immunocytochemistry of Western blots, we found the enzyme is also associated with isolated retinal synaptic fractions. Together, the data implicate the phosphatase as a prominent and integral component of many retinal synapses. Preliminary reports of this work have appeared in abstract form (4, 6, 7).

MATERIALS AND METHODS

Fertile, White Leghorn eggs obtained from a local hatchery were incubated in a forced draft incubator at 37°C. The embryos were staged according to Hamburger and Hamilton (16). Embryos between 6 and 21 d, and 2-wk-old hatchlings were used for the EM immunocytochemistry. Fractions of retinal synaptic plasma membranes (SPM) and synaptic junction complexes (SJC) were obtained from 2–10-wk-old, hatched chicks.

Calmodulin-dependent protein phosphatase was purified to homogeneity from bovine brain (30). Antisera against the enzyme was raised in a rabbit (32).

Immunocytochemistry of Fixed Tissue Slices: Animals were anesthetized and perfused through the heart for 10 min with a fixative solution containing 0.1% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate, buffered to pH 7.2 with hydrochloric acid. The anterior segment, lens, and vitreous were removed, and the posterior eyecup was continuously doused with the fixative for a further 10 min. The lower temporal quadrant of the retina was dissected out and placed in 4% paraformaldehyde in cacodylate buffer for 12–24 h, usually overnight, at 4°C. The tissue was washed briefly with a phosphate-buffered saline (PBS) pH 7.2, and immersed in a small pool of 5% agar on a piece of balsa wood. The agar was set at 4°C, and the tissue was placed in a vibratome, whereupon 40- μ m slices were cut. The slices were washed with several changes of PBS, and the agar removed from around the edges of the slices. The tissue slices were incubated for 1 h at room temperature in the primary antisera that had been diluted 1:200 with PBS. They were washed with several changes of PBS over a period of 2–3 h and then incubated in the peroxidase-conjugated Fab fragments of goat anti-rabbit IgG (Polysciences, Inc., Warrington, PA), diluted 1:600 with PBS. The slices were washed with PBS, postfixated with osmium tetroxide, dehydrated, and embedded in a mixture of Epon and Araldite. Some slices were stained en bloc after osmium fixation with 2% uranyl acetate in acetate buffer, pH 5.0. Thin sections of the slices were cut parallel to the surface of the tissue slice until a suitable balance

between good morphology and good localization of peroxidase reaction product was established. Sections of retina from embryos aged 6, 11, 13, and 17 d, and from 2-wk-old hatched chicks were analyzed for the presence or absence of product. Control slices were incubated either with the primary antisera containing an excess of antigen or without the primary antisera. Peroxidase reaction product was absent from both types of controls.

PAGE Immunoblots of Isolated Retinal Synaptic Membrane Fractions: SPM and SJC were isolated from retinal homogenates (4, 5) using a procedure adapted from Cohen et al. (3). Portions of synaptic fractions were either fixed in glutaraldehyde and osmium tetroxide, and processed for EM, or solubilized in SDS. The SDS-solubilized fractions of SPM, SJC, and antigen were subjected to PAGE on 14 \times 18 \times 0.15 cm SDS gels that consisted of 3–27% gradients. The proteins, separated by PAGE using a 3% stacking gel and a discontinuous buffer system (23), were either stained with Coomassie Blue or electrophoretically transferred from the gels onto nitrocellulose sheets according to the method of Towbin et al. (31). These nitrocellulose blots were either stained with amido black or treated sequentially with the following solutions: 5 mg/ml bovine serum albumin (BSA) in PBS, two changes for a total time of 20 min; antisera diluted 1:1,000 in PBS containing BSA (fraction V, Sigma Chemical Co., St. Louis, MO) and 0.05% Tween 20 (Sigma Chemical Co.), for 30 min; PBS, six changes for a total time of 30 min; Fab-horseradish peroxidase (Polysciences, Inc.) diluted 1:600 with PBS containing BSA and Tween 20 for 30 min; PBS, six changes for a total time of 30 min; hydrogen peroxide and diaminobenzidine (14) prepared in PBS, for 5–30 min, until reaction product appeared. The blots were washed with distilled water and dried. The peroxide reaction was done at 4°C; all other manipulations were done at room temperature. Control immunoblots either contained excess antigen in the primary antisera incubation step or were not treated with the primary antisera. Peroxidase reaction product was absent from both types of controls.

RESULTS

EM Immunocytochemistry of Fixed Retinal Slices

Detection of calmodulin-dependent protein phosphatase within the neural retina is not definitive before embryonic day 13. The peroxidase reaction product is consistently detectable, however, within photoreceptor synaptic pedicle regions at embryonic day 13 (Fig. 1), which is before the development of photoreceptor synaptic junctions (26). Reaction product is visible within the cytoplasm and is also seen as patches along the inner aspects of the undifferentiated plasma membrane of the forming synaptic pedicle. Ribbon synapses between the photoreceptor cells and horizontal cells

¹ Abbreviations used in this paper: EM, electron microscopy; SJC, synaptic junction complexes; SPM, synaptic plasma membranes.

appear between embryonic days 15 and 18 (26). Reaction product at these forming synapses is also confined to the photoreceptor presynaptic terminal (Fig. 2), and is not seen at postsynaptic sites within the neuropil of the outer plexiform layer. Within the forming photoreceptor terminals the product is associated at this time with the inner surface of the plasma membrane, the synaptic ribbons, and synaptic vesicles (Fig. 2). Photoreceptor basal junctions are not formed at this developmental time.

In the outer plexiform layer of both the embryonic day 21 and hatched chick retinas, reaction product is again seen only within the presynaptic, photoreceptor terminals (Fig. 3). Glial (Müller) cell processes between the photoreceptor cells and dendrites are devoid of reaction product (Fig. 3). At higher magnification (Fig. 3, *inset*), reaction product is now coincident with the presynaptic, paramembranous densities of basal junctions. In contrast, the product is sparse or absent from the membrane intervening between the presynaptic densities of basal and ribbon junctions (Fig. 3, *inset*). The membranes and postsynaptic densities of dendrites are not stained with the reaction product and are barely visible within the unstained sections (Fig. 3).

Calmodulin-dependent protein phosphatase is also present within putative presynaptic terminals and postsynaptic sites of forming synapses in the inner plexiform layer of the embryonic chick retina (not shown). In the mature retina of the hatched chick, the peroxidase reaction product is observed in both presynaptic and postsynaptic locations within the inner plexiform layer. Bipolar cell presynaptic terminals, recognized by the presence of a synaptic ribbon (Fig. 4), contain the

product that is associated with the synaptic ribbon and synaptic vesicles. Neuronal processes containing synaptic vesicles, typical of amacrine cell dendrites (22), in close juxtaposition to the bipolar cell terminal, do not contain product (Fig. 4). Product does label, however, the cytoplasmic side of bipolar synaptic membrane that is immediately adjacent to, and perhaps, postsynaptic to these amacrine dendritic terminals. Dendrites without vesicles, that are postsynaptic to bipolar terminals (Fig. 4) and also associated with more conventional type synapses (Fig. 5), contain reaction product. These vesicleless dendrites are typical of ganglion cells, known to receive input from both bipolar cells and amacrine cells (22). The product in these dendrites is observed in association with the postsynaptic densities at both bipolar (Fig. 4) and amacrine synapses (Fig. 5) and also with microtubules (Figs. 4 and 5).

PAGE and immunoblotting were used as a second approach to complement these EM immunocytochemical observations, and to see if calmodulin-dependent protein phosphatase was present in retinal subcellular fractions.

EM and PAGE Immunoblots of Isolated SPM and SJC Fractions

Electron micrographs of the isolated SPM fraction (Fig. 6) show that this fraction contains typical membrane-enclosed sacs, some of which bear synaptic junctions (Fig. 6, *inset*). This appears similar to SPM fractions, obtained by others, from various regions of the central nervous system. The isolated SJC fraction (Fig. 7) contains a mixture of bar-like postsynaptic densities and SJC, some of which contain rem-

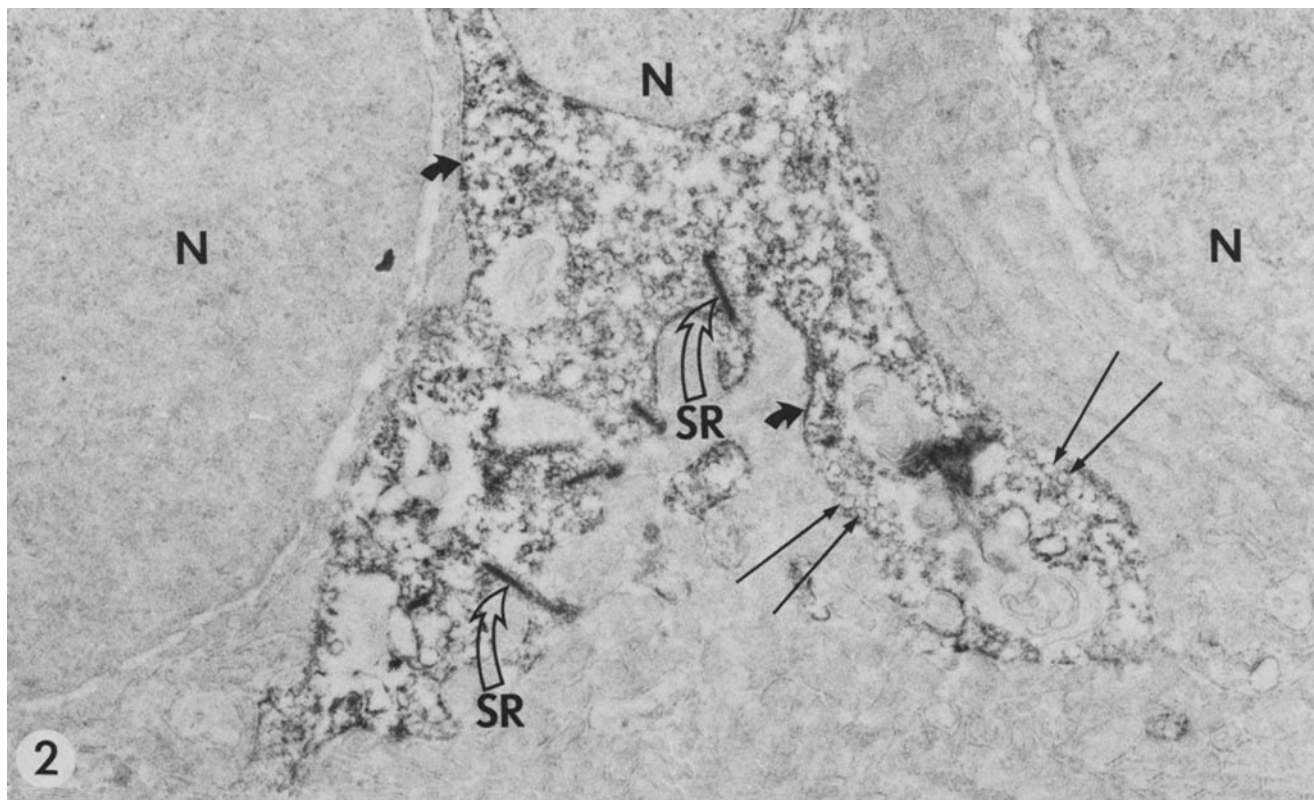


FIGURE 2 17-d unstained, embryonic retina. N, photoreceptor nucleus. Ribbon synapses between photoreceptors and dendrites are forming. The photoreceptor presynaptic ribbon (SR) appears before postsynaptic densities and ribbon junctions form before basal junctions. Synaptic vesicles are present at this time. The reaction product is present along the cytoplasmic surface of the photoreceptor plasmalemma (short arrows) and is also associated with synaptic vesicles (long arrows) and the presynaptic ribbons. $\times 27,500$.

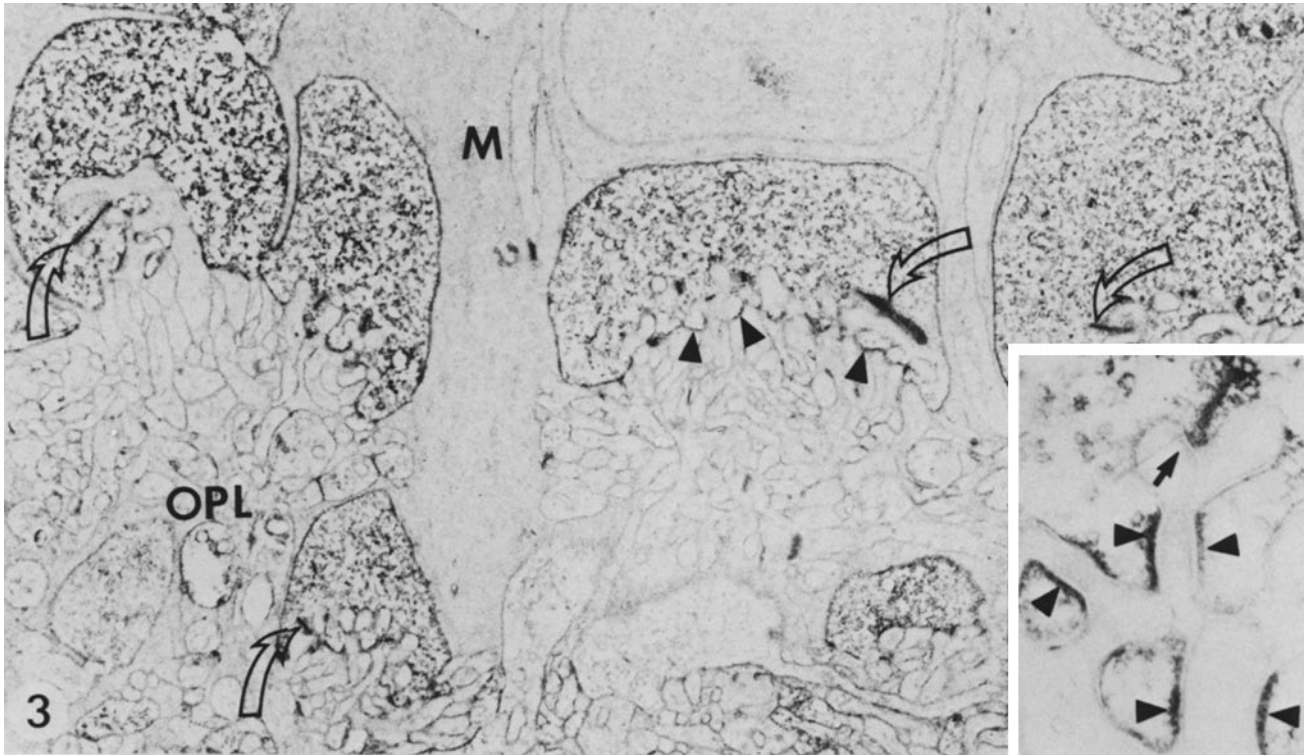


FIGURE 3 2-wk old, unstained, hatched-chick retina. OPL, outer plexiform layer. Mature ribbon and basal junctions are present. Product is still associated only with the presynaptic side of photoreceptor synapses and is not present in the intervening Müller cells (M). Product stains synaptic ribbons (open arrows) and the presynaptic densities of basal junctions (arrowheads). $\times 10,540$. (Inset) 2-wk-old, unstained hatched-chick retina. Higher magnification of photoreceptor synaptic terminal shows that the product, formerly associated with the presynaptic membrane of embryonic photoreceptor terminals (Figs. 1 and 2), is now present at both ribbon and basal junctions, and it is associated with the presynaptic ribbon (open arrow) and presynaptic paramembraneous density (arrowheads) of these two junctions. Postsynaptic densities (small arrow) where apparent do not have product associated with them. Most, but not all, synaptic vesicles together with coated vesicles are stained by reaction product. $\times 47,500$.

nants of synaptic membrane (Fig. 7, inset).

In Coomassie Blue-stained gradient gels of these two SDS-solubilized fractions (Fig. 8, lanes 4 and 5), the major synaptic proteins appear as dense bands. The four major proteins of the SJC fraction (lane 5) have relative molecular weights (M_r) of 60,000, 53,000–58,000, 45,000–46,000, and 43,000. The protein band of M_r 53,000–58,000 is enriched in the SJC fraction (lane 5) relative to the SPM fraction (lane 4). The SPM fraction (lane 4) has additional major proteins with M_r of 30,000 and 36,000.

PAGE immunoblots of the SPM and SJC (Fig. 8, lanes 6 and 7) show that they contain immunoreactive bands that correlate in position with the subunits A and B seen in the Coomassie Blue-stained gel of the purified antigen (lane 1). Lane 3 is a fraction of SPM to which some of the purified antigen has been added. This lane shows that no additional bands can be detected and that the intensity of bands corresponding to the phosphatase subunits is increased relative to the SPM (lane 4) and SJC (lane 5) fractions. This increased intensity helps to demonstrate that the lower molecular weight subunit (labeled *b*) of the phosphatase is the middle band of the last three bands seen in lanes 4 and 5 (SPM and SJC). The immunoreactive bands due to the subunits of the phosphatase and faint immunoreactive bands seen interposed between the phosphatase subunits in lanes 6 and 7 of SPM and SJC are not present in control blots that were either incubated in PBS in place of the primary antisera (not shown) or incubated in the primary antisera containing an excess of

the purified antigen (SPM, lane 8; SJC, lane 9). This suggests that the immunoreactive bands interposed between the subunits are probably proteolytic fragments of the subunit A. Such fragments are sometimes observed in the purified antigen. It should be noted, however, that the preparative procedures for the purified antigen and the retinal fractions are different, and consequently minor differences are to be expected. In some antigen preparations, a major band with M_r of 58,000 is detected (not shown) which may be a degraded or otherwise altered product of the subunit A as has been reported elsewhere (28, 31).

DISCUSSION

This study confirms the association of calmodulin-dependent protein phosphatase with postsynaptic densities (32, 36) and the existence of the enzyme in the retina (29, 35). In addition, the report extends these earlier observations, demonstrating at the EM level the association of the enzyme with presynaptic as well as postsynaptic elements in the chick retina. The presence of the enzyme in photoreceptor, bipolar, and ganglion cells and its absence from the more radially disposed horizontal and amacrine cells is demonstrated. In identified synapses the enzyme was seen either in presynaptic or postsynaptic, or in one case both pre- and postsynaptic sites. Also, the subcellular fractionation data presented here supports the EM findings of an association of this enzyme with SPM and SJC.

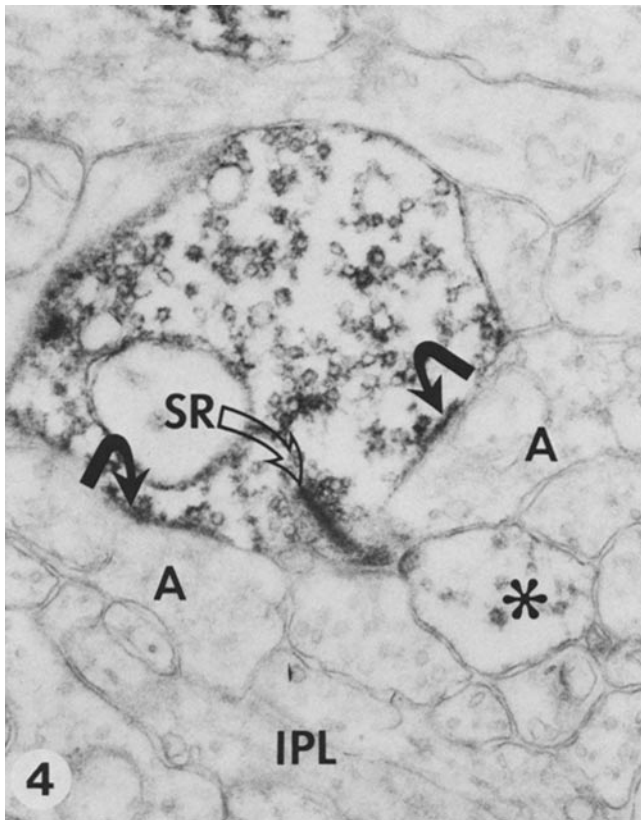


FIGURE 4 2-wk-old, stained hatched-chick retina. *IPL*, inner plexiform layer. Synaptic ribbon (*SR*)-containing bipolar terminal contains reaction product. A dendrite (*) postsynaptic to the bipolar terminal that does not contain synaptic vesicles is stained with reaction product. Thin membrane densities (curved arrows) within the bipolar terminal stained with reaction product appear to be postsynaptic to vesicle-containing neuronal profiles (*A*) that synapse on the bipolar terminals. $\times 45,600$.

During development, the enzyme is shown to be present before the formation of recognizable synapses. The particular aspect of neurogenesis that signals the initiation of phosphatase synthesis remains to be determined, but it is likely that this event occurs just before synaptogenesis. During development, the enzyme becomes associated with some of the synaptic structures. This together with the demonstrated presence of the enzyme in isolated synaptic membranes is in agreement with radioimmunoassay studies of developing rat cerebrum. These latter studies demonstrate that the level of antigen increases in both a soluble and particulate fraction during development, and the particulate fraction contains a higher level of the antigen on the basis of protein.

While the precise localization of calmodulin within synaptic terminals has not been demonstrated, its presence is indicated by other types of studies and is also indirectly supported by the present study. DeLorenzo (9) has suggested that calmodulin-dependent phosphorylation of synaptic vesicle proteins may be a mechanism that aids in the fusion of vesicles with the presynaptic membrane during exocytosis of transmitter. The study here has shown an association of the calmodulin-dependent protein phosphatase with synaptic vesicles at the EM level. It remains to be established if this is a functional association because the phosphatase is likely to be present in

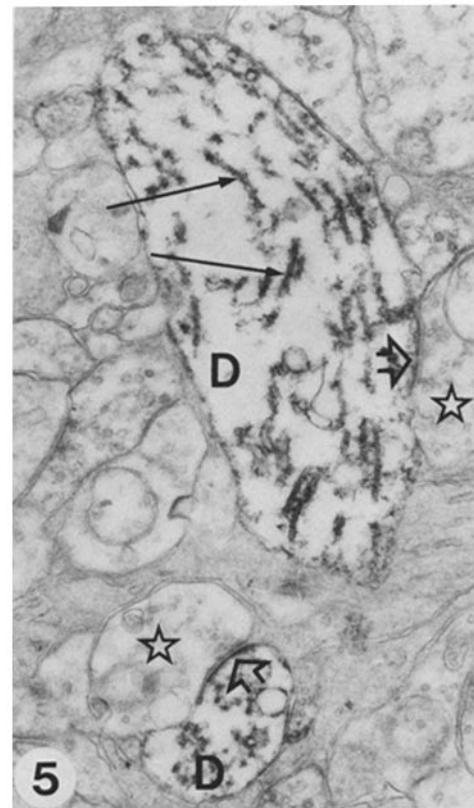
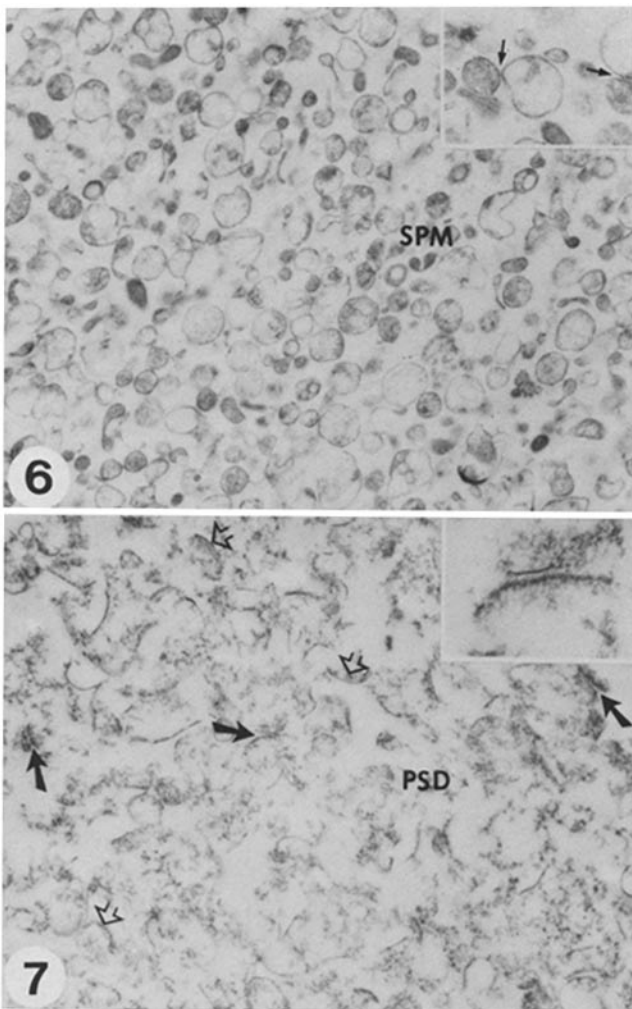


FIGURE 5 2-wk-old, stained, hatched chick retina. Inner plexiform layer. *D*, dendrite. Reaction product in dendrites is associated mostly with postsynaptic densities (open arrow) and also with neurotubules (long arrow). The presynaptic elements of those more conventional type synapses (☆) do not contain product. $\times 28,500$.

both cytoplasmic, soluble forms and membrane-bound, insoluble forms (29). Thus, the possibility exists that the association of enzyme with vesicles and microtubules seen here is due to precipitation of the cytoplasmic enzyme onto these components during fixation. Nevertheless, it is clear that the phosphatase is a presynaptic component of some synapses and its presence presynaptically raises the possibility that it is involved in the electrochemically mediated transfer of information between neurons. While synapsin-1, the substrate for cyclic nucleotide-dependent and calmodulin-dependent protein kinase, is associated with synaptic vesicles (8, 19), it is not known if this is also the endogenous substrate for the calmodulin-dependent protein phosphatase shown here. The phosphatase can dephosphorylate synapsin-1, however, under *in vitro* conditions (20).

Kennedy et al. (18) and Grab et al. (11-13) have shown that a calmodulin-dependent protein kinase constitutes a major component of the postsynaptic densities, and more recent studies (10) suggest that the major protein of the postsynaptic densities of the brain is homologous with the major calmodulin-binding subunit of a calmodulin-dependent protein kinase.

The present study suggests that the calmodulin-dependent protein phosphatase is a prominent component of the retinal neuropil because it is present in many retinal synapses as either a presynaptic or postsynaptic component and because the immunoreactive band of subunit A coincides with a



FIGURES 6 and 7 (Fig. 6) Retinal synaptic plasma membrane (SPM) fraction showing typical membranous sacs. Section taken from middle of the pellet. Smaller and larger sacs are present at higher and lower positions in the pellet, respectively. $\times 41,800$. (Inset) This fraction also contains SJC (arrows). $\times 64,600$. (Fig. 7) Retinal synaptic junction complex fraction. This fraction contains a mixture of postsynaptic densities (open arrows) and SJC (closed arrows). Membrane remnants are associated with some junctions, whereas others appear completely demembranated. $\times 38,000$. (Inset) Higher magnification of typical synaptic junction. $\times 108,300$.

prominent protein band in gels stained with Coomassie Blue. While the phosphatase is associated at the EM level with some postsynaptic densities and microtubules, it remains to be seen which of these are functional associations. Although tubulin has been associated with postsynaptic densities and synaptic vesicles in other studies (17), it is not known if this would serve as a substrate for the phosphatase. The endogenous substrate for calmodulin-dependent protein phosphatase in both presynaptic and postsynaptic sites remains to be determined.

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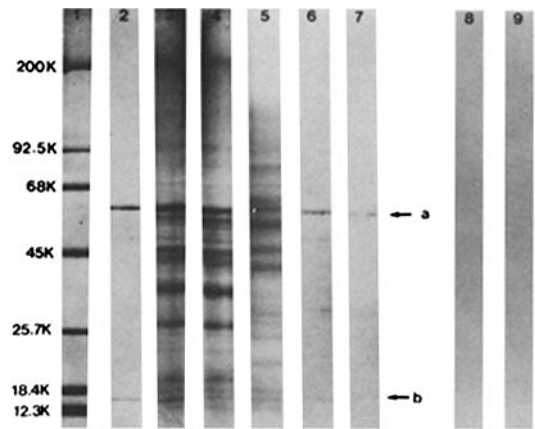


FIGURE 8 Coomassie Blue (cb)-stained polyacrylamide gels and antisera (as)-treated PAGE immunoblots of retinal SPM and SJC fractions. Lane 1, cb-stained gel of standards with molecular weights in kilodaltons indicated to the left of the lane. Lane 2, cb-stained gel of purified antigen (2 μg protein). Lane 3, cb-stained gel of SPM (80 μg protein) to which 2 μg purified antigen was added before electrophoresis. Lane 4, cb-stained blot of SPM (80 μg protein). Lane 5, cb-stained gel of SJC (80 μg protein). Lane 6, as-treated blot of SPM (80 μg protein). Lane 7, as-treated blot of SJC (80 μg protein). The arrows to the right of this lane (a and b) indicate the positions of the subunits A and B of the phosphatase. Lane 8, as-treated blot of SPM (80 μg protein) incubated with primary antisera containing an excess of the purified antigen. Lane 9, as-treated blot of SJC (80 μg protein) incubated in the primary antisera containing an excess of the purified antigen.

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