Identification of a Synaptic Vesicle-Specific Membrane Protein with a Wide Distribution in Neuronal and Neurosecretory Tissue

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ABSTRACT Two different monoclonal antibodies, characterized initially as binding synaptic terminal regions of rat brain, bind a 65,000-dalton protein, which is exposed on the outer surface of brain synaptic vesicles. Immunocytochemical experiments at the electron microscope level demonstrate that these antibodies bind the vesicles in many different types of nerve terminals. The antibodies have been used successfully to purify synaptic vesicles from crude brain homogenates by immunoprecipitation onto the surface of polyacrylamide beads. The profiles of the structures precipitated by these beads are almost exclusively vesicular, confirming the vesicle-specificity of the antibodies. In SDS gels, the antibodies bind a single protein of 65,000 daltons. The two antibodies are not identical, but compete for binding sites on this protein.

Immune competition experiments also demonstrate that the antigenic components on the 65,000-dalton protein are widely distributed in neuronal and neural secretory tissues. Detectable antigen is not found in uninnervated tissue—blood cells and extrajunctional muscle. Low levels are found in nonneural secretory tissues; it is not certain whether this reflects the presence of low amounts of the antigen on all the exocytotic vesicles in these tissues or whether the antigen is found only in neuronal fibers within these tissues. The molecular weight and at least two antigenic determinants of the 65,000-dalton protein are highly conserved throughout vertebrate phylogeny. The two antibodies recognize a 65,000-dalton protein present in shark, amphibia, birds, and mammals. The highly conserved nature of the determinants on this protein and their specific localization on secretory vesicles of many different types suggest that this protein may be essential for the normal function of neuronal secretory vesicles.

Synaptic vesicles mediate the release of neurotransmitters in the nervous system and are essential for normal interaction between neurons (16, 17). Similar vesicles in other cells release polypeptide hormones, digestive enzymes, and a wide variety of secreted proteins (cf. reference 27). In fact, all cells contain membrane vesicles involved in fusion events, either intracellularly or at the plasmalemma. They are involved in biogenesis of the surface membrane and lysosomes (cf. references 35, 39), and mediate recycling and endocytosis (cf. reference 13). The different vesicle classes must not only have different membrane and internal contents reflecting their different metabolic and storage functions; they must also have different constituents responsible for directing them to appropriate portions of the cell and ensuring that they fuse with appropriate organelles.

One approach to understanding the biochemical basis of vesicle function has been to purify vesicles and study the molecules present in these preparations. The vesicles that have been purified and studied in this way include pancreatic vesicles (46), platelet and mast cell granules (9, 48), parotid vesicles (7, 8), chromaffin vesicles from the adrenal medulla (cf. reference 52), large adrenergic vesicles from sympathetic nerve trunks (34), cholinergic vesicles from electric fish (6), and vesicles from the central nervous system (cf. reference 1; 47). In each case characteristic enzymatic activities and protein constituents have been identified in the vesicle membrane. Some of these are highly enriched in vesicles compared to other membranous preparations (cf, 1, 50). Each vesicle class has also been shown to have specific internal contents—including,

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in individual cases, transmitters, zymogens, peptides, enzymes, proteoglycans, and proteolipids (cf. references 7, 19, 31, 42, 52). Comparative studies between these few highly purified preparations have seldom been done. In most cases, the uncertain or incomplete purity of the vesicle fractions would compromise the results (cf. 31).

One approach to comparing different vesicle populations and understanding the biochemical bases of their specific functions is immunological. Specific antibodies can reveal the presence of even minor constituents, can be used in immunohistochemical studies to identify the subcellular distribution of particular antigens, and can identify those antigens that are shared between vesicles containing different transmitters or conserved during evolution. For example, biochemical and immunological methods have provided strong evidence that many of the individual proteins in chromaffin granules are also in vesicles isolated from sympathetic neurons (cf. 2, 34), and some of these antibodies have been used also to identify adrenergic neurons in the central nervous system (cf. 45). An antiserum against a highly purified preparation of elasmobranch cholinergic vesicles has been used to show that antigens present in the fish vesicles are conserved in the vertebrate phylogeny and are found in a subclass of mammalian nerve terminals, including adrenergic, cholinergic, and some peptidergic neurons (21). These experiments revealed an unsuspected relationship between these terminals, but did not identify the antigenic molecules or determine whether the same antigen was being detected in each class of terminals. Several years ago we initiated a series of attempts to identify molecules highly enriched in brain synapses by preparing monoclonal antibodies from the spleens of mice immunized with synaptic densities, containing the pre- and postsynaptic surface membranes and material between the membranes. Unexpectedly, two of these monoclonal antibodies have proved to be directed against a protein that is highly enriched in synaptic vesicles. Here we characterize these antibodies and the protein which they bind. We present evidence that this protein is highly conserved in the vertebrate phylogeny and is present in many different types of secretory vesicles, suggesting that it may be required for normal vesicle activity. Since the antibody molecules bind determinants on the outside of synaptic vesicles, they can be used in conjunction with biochemical procedures to purify these vesicles from brain homogenates and should be useful in preparing more homogeneous preparations of vesicles than are possible with biochemical methods alone. They should also be useful in identifying the contents of vesicles from microdissected brain regions, including putative neurotransmitters, and in tracing the pathways of vesicle membrane biogenesis and cycling.

MATERIALS AND METHODS

Isolation of Hybridoma Secreting Cell Lines

Rat brain synaptic membranes were prepared by the procedure of Jones and Matus (29). Synaptic junctional complexes were prepared from these by the procedure of Wang and Mahler (51). BALB/c mice were immunized with 100 μg of synaptic junctional protein in complete Freund's adjuvant, i.p. on day 0, and in saline, i.v., on day 10. Only mice that gave a strong positive immune response were injected on day 10. Spleens were removed on day 13 and fused with NS-1 myeloma cells using polyethylene glycol (PEG) in the procedure of Herzenberg et al. (15). Fusions were distributed into 500 microwells and hybrids were selected with hypoxanthine/aminopterin/thymidine (HAT) medium (15). When the medium turned yellow, individual wells were assayed in a solid-phase radioimmune assay, using rat brain synaptic membranes as the solid-phase antigen and [^{125}I]labeled affinity purified goat-anti-mouse-kappa chain immunoglobulin as an indirect probe (14, 32). The positive wells were screened against rat liver, kidney, spleen, and thymus membranes in the same assay. Those wells that appeared to be brain-specific by these tests were adapted to culture and recloned with micromanipulation, using fluorescent beads bound to synaptic membrane (40).

Preparation of Antibodies

Culture supernates were prepared from overgrown cultures of hybridoma cell lines. Debris was removed by centrifugation at 10,000 *g* for 30 min. Supernates were stored at −20°C and were filtered through a 0.45-μm filter before use.

Ascites fluid was prepared by i.p. injection of cultured hybridoma cells into BALB/c mice primed 3–60 d previously with 0.5 ml of pristane. In some cases, the hybridoma cells were first injected subcutaneously and the resulting tumors were used for i.p. injections. Fluid from obviously swollen mice was collected into 0.01 M Na-citrate by i.p. puncture. After cells and debris were removed by centrifugation at 10,000 *g* for 30 min, the supernate was precipitated with 50% saturated ammonium sulfate, pH 5.5, resuspended in 0.9% NaCl, 0.01 M $NaPO_4$, pH 7.4, dialyzed against the same buffer, stored at −80°C at a concentration of 20–30 mg/ml, and filtered through a 0.45-μm filter before use.

Immunohistochemistry

Light and electron microscopy were done on sections prepared as described by Vaughn et al. (49). In detail, for light microscopy, the animal was anesthetized with Nembutal, perfused briefly with 150 mM NaCl, and perfused thoroughly with 4% paraformaldehyde in 0.12 M Millonig's buffer at 4°C. After storage of the animal for 60 min at 4°C, the tissues of interest were removed and cut into 2- to 3-mm slices with a razor blade. After 3-h incubation at 4°C in the same fixative, the slices were washed with four changes of 300 ml of 0.12 M Millonig's buffer over a 3-h period and immersed in 30% (wt/vol) aqueous sucrose overnight. Frog tissues were prepared by an abbreviated protocol. After removal from unperfused animals, tissues were immersed for 60 min in fixative and carried through the same subsequent steps in the procedure. In each case, 8- to 10-μm frozen sections were prepared on a cryostat.

For electron microscopy (EM), the anaesthetized animal was perfused briefly with 150 mM NaCl at 37°C and extensively with ice-cold 0.1–0.25% glutaraldehyde, 4% paraformaldehyde in 0.12 M Millonig's buffer. After storage for 60 min at 4°C, tissues were removed and cut into 2- to 3-mm sections with a razor blade. After 2–3 h, further incubation in fixative at 4°C, 50-μm vibratome sections were prepared and washed in 3–4 changes of 0.12 M Millonig's buffer over 12–16 h.

When necessary, endogenous peroxidase was suppressed in peripheral tissues by treatment with $NaIO_4$ and $NaBH_4$ (43). Before use, goat-anti-mouse serum, conjugate, and rat peroxidase antiperoxidase complex (PAP) were adsorbed with rat liver powder. The immunocytochemical procedures were initiated by incubating sections for 2–3 h with 3% normal goat serum (NGS) in phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM $NaPO_4$, pH 7.4). To improve penetration, 0.1% Triton X-100 was included with some samples processed for light microscopy. Then monoclonal antibodies at concentrations of 5–10 μg/ml were applied as undiluted, clarified culture supernates for 6–16 h (16 h for EM). Sections were washed with six changes of 1% NGS in PBS over 3 h (6 h for EM), followed by 2-h incubation with 3% NGS in PBS (3 h for EM). Sections were then incubated with 10% goat-anti-mouse serum (N. L. Cappel Laboratories, Inc., Cochranville, Pa.), 1% NGS in PBS for 3 h (16 h for EM). Sections were washed for 2 h (5 h for EM) with four changes 1% NGS in PBS, 60 min with 3% NGS in PBS, and 3 h (16 h for EM) with rat PAP prepared as described by Sternberger (44) (40 μg/ml peroxidase). Afterwards, sections were washed for 3 h with six changes of PBS and for 1 h (2 h for EM) with two changes of 0.05 M Tris-Cl, pH 7.6. Occasionally, affinity purified goat-anti-mouse IgG-peroxidase conjugate, prepared in our laboratory by Mr. E. Outwater with the heterobifunctional cross-linking reagent N-succinimidyl-3(Z-pyridyldithio)proponate (SPDP) (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), was used in place of goat-anti-mouse serum and rat PAP.

The frozen sections for light microscopy were incubated with freshly prepared and filtered 0.36 mg/ml diaminobenzidine (DAB), 0.05 M Tris-Cl, pH 7.6, 0.01% H_2O_2, for 7–10 min at 20°C. The vibratome sections for EM were agitated in the dark for 30 min at 0°C with 0.36 mg/ml DAB, 0.05 M Tris-Cl, pH 7.6. They were then incubated for 7–10 min at 0°C with fresh 0.36 mg/ml DAB, 0.01% H_2O_2, 0.05 M Tris-Cl, pH 7.6. After reaction, both sets of sections were washed with three to four changes of PBS (2 h for frozen sections; 16 h for vibratome sections).

Before examination, frozen sections were dehydrated and clarified in graded ethanols and xylene. For EM, the sections were fixed with 2% OsO_4 in PBS, washed in PBS, dehydrated with graded alcohols and propylene oxide, and embedded in Araldite. After embedding, 3-μm sections were prepared and examined. Sections with good preservation and development of peroxidase products were re-embedded in Araldite. 60-to 80-nm sections were prepared for examination in the EM.

Three controls were run in parallel on tissue sections. Fresh culture medium, normal mouse serum, or monoclonal serum 9 was substituted for serum 30. No detectable binding was seen with culture medium or normal mouse serum, while serum 9, which binds galactose moieties on terminal glycolipids or glycoproteins (unpublished observations), exhibited a very specific binding to white matter. A purified antigen was not available to remove antibody molecules, but the monoclonal antibodies were the only immunoglobulins detectable in the culture supernates used for immunocytochemistry.

Immunoprecipitation of Organelles

200 mg of hydrophilic polyacrylamide beads (Bio-Rad Laboratories, Richmond, Calif.), nominally 5–10 μm in diameter, were coupled to 5 mg of protein A (Pharmacia Fine Chemicals) in 20 ml 0.003 M KPO_4 buffer, pH 6.3, using 40 mg of [1-ethyl-3 (3-dimethlaminopropyl) carbodiimide-HCl], following the procedure supplied by Bio-Rad Laboratories (24). 3 mg of protein A was actually coupled to the 200 mg of acrylamide beads with this procedure.

For immunoprecipitation, we made a very crude lysed rat brain synaptosome preparation by following the procedure of Jones and Matus (29) through the hypotonic lysis step, but without subsequent separation on sucrose gradients of the different membrane and organelle fractions. The solution from three rat brains was adjusted to 30 ml of 0.12 M sucrose, 75 mM NaCl, 5 mM $NaPO_4$, pH 7.4.

For each immunoprecipitation, 4 ml of membrane solution was mixed for three h at 2°C with ~0.8 mg of antibody (50% saturated $[NH_4]_2SO_4$ precipitate from mouse ascites fluid). The solution was diluted with H_2O to 0.09 M sucrose and pelleted at 300,000 *g* for 90 min. Pellets were resuspended in 2 ml of 0.32 M sucrose by hand homogenization in a glass-teflon homogenizer. After dilution with 2 ml of 75 mM NaCl, 5 mM $NaPO_4$ (pH 7.4), 10 mg/ml bovine serum albumin (BSA), membrane aggregates were removed by centrifugation at 700 *g* for 10 min. The supernate was mixed with 5 mg of protein A–acrylamide beads for 70 min at 2°C, and centrifuged at 300 *g* for 10 min. The beads were washed three times with isotonic sucrose–PBS (160 mM sucrose, 75 mM NaCl, 5 mM $NaPO_4$, pH 7.4) and three times with PBS (150 mM NaCl, 10 mM $NaPO_4$, pH 7.4) by centrifugation at 300 *g* for 10 min. Samples of the pelleted beads and pelleted crude membrane fraction were fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.2, for 1 h at 0°C. After brief washing in 0.1 M Na-cacodylate, pH 7.2, samples were postfixed in 2% OsO_4 in the same buffer for 1 h at 0°C. Samples were then washed twice with cold 0.9% NaCl and stained with 0.5% uranyl acetate, 0.9% NaCl, for 1 h at 20°C. Finally, samples were dehydrated in graded ethanols, embedded in Araldite, sectioned, and examined in the EM.

Quantitative Measurement of Antigen in Different Tissues

Aliquots of 76 ng/ml of ascites fluid 48 in 5% newborn calf serum in PBS (a dilution chosen to be limiting in the assay) were incubated with different concentrations of homogenized tissues for 12 hours at 4°C. The membranes were pelleted at 200,000 *g* for 40 min in an airfuge. The residual antibody in the supernate was measured in the solid-phase radioimmune assay (32). The amount of tissue protein needed to adsorb 50% of the binding was recorded from inhibition curves.

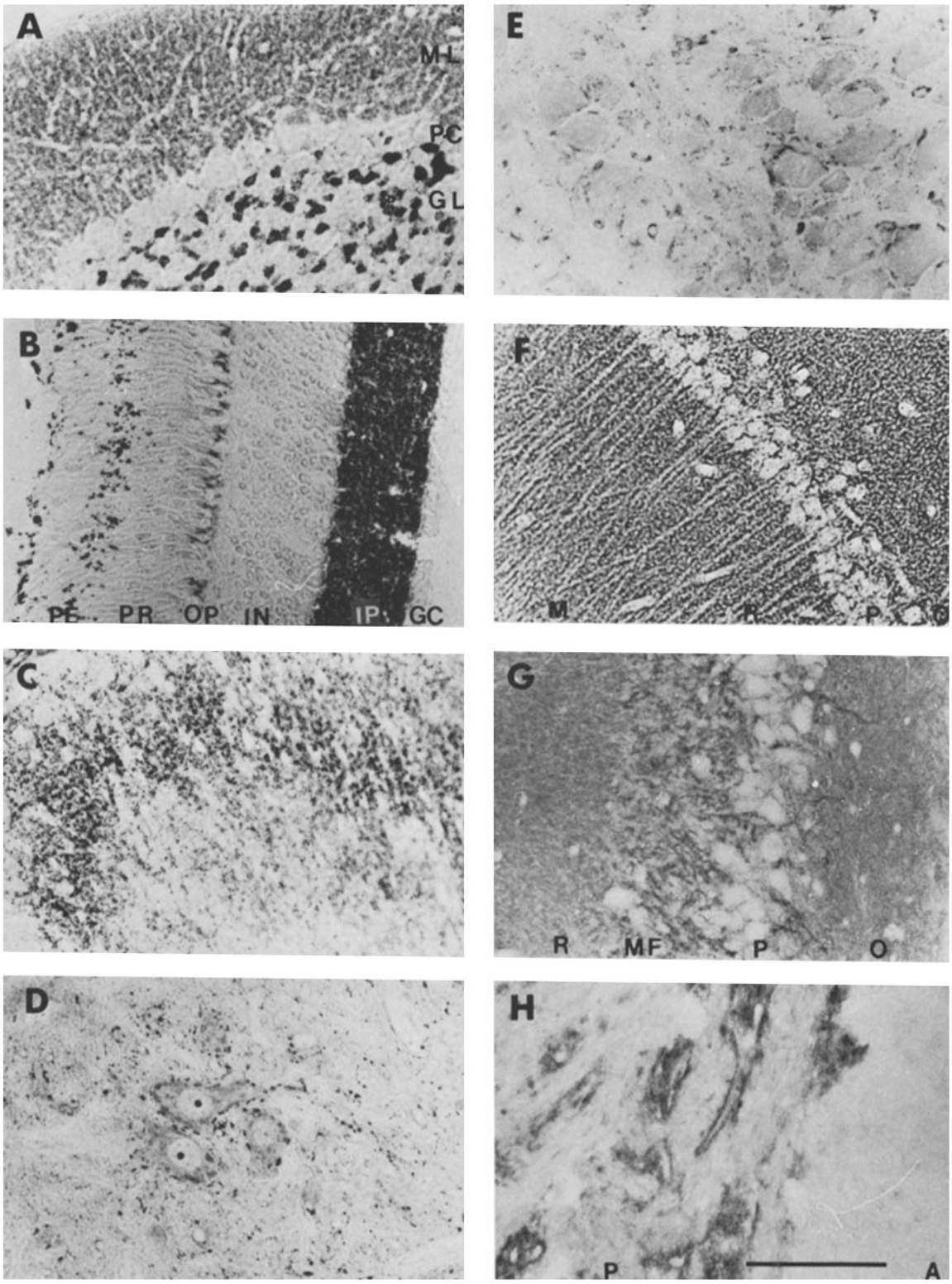
RESULTS

Localization to Nerve Terminals and Synaptic Vesicles

By procedures described in Materials and Methods, a series of hybridoma cell lines were isolated that secreted antibodies that bound to brain synaptic junctions much more than to liver, kidney, spleen, and thymus membrane preparations. Examples of the immunohistochemical binding patterns of serum 30, one of the two antisera discussed in this paper, are shown in Fig. 1. In sections of the cerebellum (Fig. 1 *a*), binding to the large glomeruli in the granule cell layer is particularly prominent, but tiny punctate binding regions are also visible in the molecular layer, where a variety of much smaller nerve terminals are found. The antigen is found in both of the synaptic regions of the retina (Fig. 1 *b*), the inner and outer plexiform layers. The comparative density of reaction product corresponds roughly to the density of synapses in the two regions. The retinal layers that do not contain synapses are not stained. In the spinal cord, small punctate depositions of reaction product are visible throughout the gray matter and are particularly dense in the substantia gelatinosa (Fig. 1 *c*). The axosomatic synapses on motoneurons in the ventral gray matter are also stained (Fig. 1 *d*). In the sympathetic ganglion, reaction product is localized to punctate areas near the large principal neuron cell bodies, where the majority of axodendritic synapses in that ganglion are located. Every layer of the hippocampus contains synapses, and punctate spots of reaction product are visible in every layer of CA1 (Fig. 1 *f*) and CA3 (Fig. 1 *g*). In the region where large mossy fiber terminals are located in CA3, correspondingly large depositions of reaction product are visible (Fig. 1 *g*). Serum 30 also binds to regions in the posterior pituitary around capillaries (Fig. 1 *h*) in a distribution consistent with the distribution of nerve terminals in that gland. In summary, serum 30 binds a wide variety of nerve terminals in the central and peripheral nervous systems; there is no terminal region that has been examined that does not react with this antiserum. Serum 48 has not been tested so extensively but produces identical patterns in the cerebellum and spinal cord, the two tissues examined (data not shown). Sections of each tissue in which normal mouse serum was substituted for serum 30 were carried through the reaction sequence in parallel and did not contain visible reaction product.

To more definitively identify the location of the antigen bound by serum 30, we examined similarly treated sections from the cerebellum in the EM (Fig. 2). Binding was seen to a variety of nerve terminals–mossy fiber terminals and Golgi complex type II terminals in the granule cell layer (Fig. 2 *a*), and a myriad of small terminals in the molecular layer (Fig. 2 *c*), including parallel fiber terminals on Purkinje cell dendrites. While reagents penetrate only short distances into the 50- to 100-μm sections used for PAP immunohistochemistry, virtually every terminal has reaction product in thin sections prepared from the surface region of the vibratome sections. Examination of several hundred fields in the EM convinced us that no identifiable class of terminals was unstained. Similarly, all synaptic layers in other sections of the nervous system visible by light microscopy appear to have reaction product (Fig. 1). Serum 48 also stains all visible terminals in the cerebellum (data not shown).

Higher power EM micrographs of mossy fiber and parallel fiber terminals in Fig. 2 *b* and *d* show that the peroxidase reaction product is concentrated on the outer surface of synaptic vesicles. Some reaction product is also found on other membrane structures facing these vesicles, e.g. mitochondria and plasmalemma. In EM micrographs of synaptosomes, immunoreactivity is restricted to synaptic vesicles and occasional patches of plasmalemma or mitochondria adjacent to these vesicles (data not presented). Since reaction product is seen only on those faces of these other organelles that are closely apposed to vesicles, migration of reaction product seems likely to explain all the product on the mitochondria and most of that on the plasmalemma. In principle, one would expect to find some vesicle membrane antigens in the plasmalemma, and this can be documented in the adrenal medulla. In Fig. 3 *a*, a low power micrograph of adrenal gland, the DAB reaction product is found in medulla, but not cortex. In EM micrographs of the adrenal medulla (Fig. 3 *b*), reaction product is found on the membrane of chromaffin granules and also on those portions of the plasmalemma that face sinuisoidal spaces. Reaction product is not seen on the other portions of the plasmalemma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even though the lateral membranes are also clearly apposed to chromaffin granules. For this reason, we believe that this represents real binding of the monoclonal antisera to the plasmalemma of these cells. In these sections, the reaction product is found on the outer surface of vesicles and the inner surface of the plasmalemma, consistent with the results in cerebellar terminals. Serum 30 and 48 bind the same structures (comparative data not shown).

Immunoprecipitation of Synaptic Vesicles

To obtain more definitive evidence for the localization of these antigens to synaptic vesicles, we used the procedures of Ito and Palade (25) to see which organelles were precipitated by these antisera. A crude nerve terminal preparation was lysed with a hypotonic solution to release internal organelles. The mixture of membranes was incubated with a monoclonal antibody and then precipitated after brief mixing with protein A–coupled acrylamide beads. After several washes, the beads were fixed, sectioned, and examined in the EM. In Fig. 4*a* is the lysed terminal preparation that was incubated with the monoclonal antibody. Before the pellet was fixed, the membranes were diluted with an equal volume of buffer and mixed thoroughly. This insures that the various membranes are distributed randomly in the pellet. A mixture of partially lysed synaptosomes, mitochondria, vesicles, and debris is evident. Fig. 4*b* is a representative photograph of the profiles precipitated by normal mouse serum. Only an occasional piece of membrane is found on the surface of the polyacrylamide beads. In experiments with monoclonal serum not directed against membrane antigens, we found even fewer profiles on the beads (data not shown). In contrast, when serum 30 is used (Fig. 4*c* and *d*) large numbers of uniformly sized profiles are found on the surface of the beads. Virtually all the attached membranous materials have the small uniform size of synaptic vesicles. Plasmalemma profiles are rare; mitochondrial profiles are absent. The structures seen in sections of total and immunologically purified membranous material are tabulated in Table I. Most of the purified organelles are synaptic vesicles. The other profiles in the purified fraction include small uncharacterized structures, whose size makes them likely to be disrupted synaptic vesicles, and larger membranes, probably derived from the plasmalemma. Since the contaminating structures are on average larger than synaptic vesicles, individual organelles have a higher probability of being visualized in sectioned material. Hence their frequency, but not their mass, is overestimated in Table I. It is clear from this table that a single immunoprecipitation step is capable of significantly purifying synaptic vesicles from a crude starting preparation. The amount of nonvesicular material is reduced 30-fold by this single step. The major limitation to the efficacy of this purification may be the presence of the vesicle antigen in inverted synaptic plasma membranes.

To demonstrate that intact vesicles can be precipitated by these monoclonal antibodies, we incubated a rat pheochromocytoma cell line, PC12, which contains adrenergic vesicles, with [^{3}H]norepinephrine (NE) before homogenizing the cells (18) and precipitating the organelles via the externally exposed antigen. The efficiency of this precipitation has been estimated in Table II. It has previously been shown that vesicular but not cytoplasmic [^{3}H]NE can be retained in appropriate conditions on Millipore filters (41), presumably because vesicle proteins bind the entire vesicle to nitrocellulose. Using this assay to estimate the [^{3}H]NE remaining in the vesicles after homogenization, we found that 40% of the counts were retained in vesicles. Over the subsequent 4 h occupied in the various immunoprecipitation steps, 55% of these counts were released from vesicles. Antibodies did not increase the rate of release. 95% of the [^{3}H]NE remaining in vesicles could be immunoprecipitated on acrylamide beads, so immunoprecipitation of vesicles is efficient. The precipitation of vesicle contents is limited

FIGURE 1 Immunocytochemical localization of serum 30 immunoreactivity in the nervous system. Light microscopy of representative 8–10 μm formaldehyde-fixed, frozen sections that have been incubated with serum 30 and rat PAP complex or peroxidase conjugate are shown in this figure. The peroxidase reaction product appears dark. Unless indicated, sections are not counterstained. Control experiments, omitting the monoclonal antiserum and substituting normal mouse serum, were done on sections of each tissue at the same time. Visible reaction product was not seen in these sections. (*A*) Rat cerebellar cortex, showing granule cell layer (*GL*) at bottom, Purkinje cell layer (*PC*) in center, and molecular layer (*ML*) at top. The peroxidase reaction product is abundant in synaptic regions, staining the small synapses in the molecular layer with small, punctate depositions and the large synaptic glomeruli in the granular cell layer with large reaction-product depositions. The white matter (not shown) contained no reaction product. (*B*) Frog retina, showing the pigment epithelium (*PE*) at the left, the photoreceptors (*PR*), outer nuclear layer (not abbreviated), outer plexiform layer (*OP*), inner nuclear layer (*IN*), inner plexiform layer (*IP*), and ganglion cells (*GC*). Peroxidase product is seen in the two synaptic layers—the outer and inner plexiform layers. The dark product in the pigment epithelium is endogenous melanin and is seen in control sections. Although not apparent in black-and-white photographs, the two deposits are easily distinguishable, since the melanin is black and the peroxidase product is brown. (*C*) Rat thoracic spinal cord, showing the substantia gelatinosa. Small depositions of peroxidase reaction product are seen throughout the spinal cord gray matter, particularly in synapse-rich areas such as the crescent-shaped substantia gelatinosa shown in this section. (*D*) Rat thoracic spinal cord, showing the large motoneurons in the ventral gray matter. This section was lightly counterstained with toluidine blue to reveal the motoneuron somas. Reaction product in the axosomatic synapses on the motoneurons is particularly dramatic. (*E*) Frog paravertebral sympathetic ganglion (T9 or T10), showing the large principal sympathetic neuron cell somas. Peroxidase reaction product is localized to punctate areas between the cell somas, as is expected in this ganglion where the majority of synapses are formed on dendrites. The light brown coloring of the cell somas was also seen in controls. (*F*) Rat hippocampus, CA 1 area, showing the stratum moleculare (*M*) at the left, the stratum radiatum (*R*), stratum pyramidale (*P*), and stratum oriens (*O*). Many different types of synapses are found between cells in these areas and punctate depositions of peroxidase reaction product are also seen throughout the section. (*G*) Rat hippocampus, CA 3 area, showing the stratum oriens (*O*) at the right, and the stratum pyramidale (*P*), mossy fiber terminals (*MF*), and stratum radiatum (*R*). Small depositions of reaction product, corresponding to the synaptic terminals, are found throughout this section. Somewhat larger, 3- to 6-μm long terminals, formed by mossy fibers, and correspondingly larger depositions of reaction product are found in the mossy fiber area (*MF*). (*H*) Rabbit pituitary, showing portions of the posterior (*P*) and anterior (*A*) lobes. Peroxidase reaction product is evident in the posterior lobe in regions surrounding the capillaries. Bar, 100 μm.

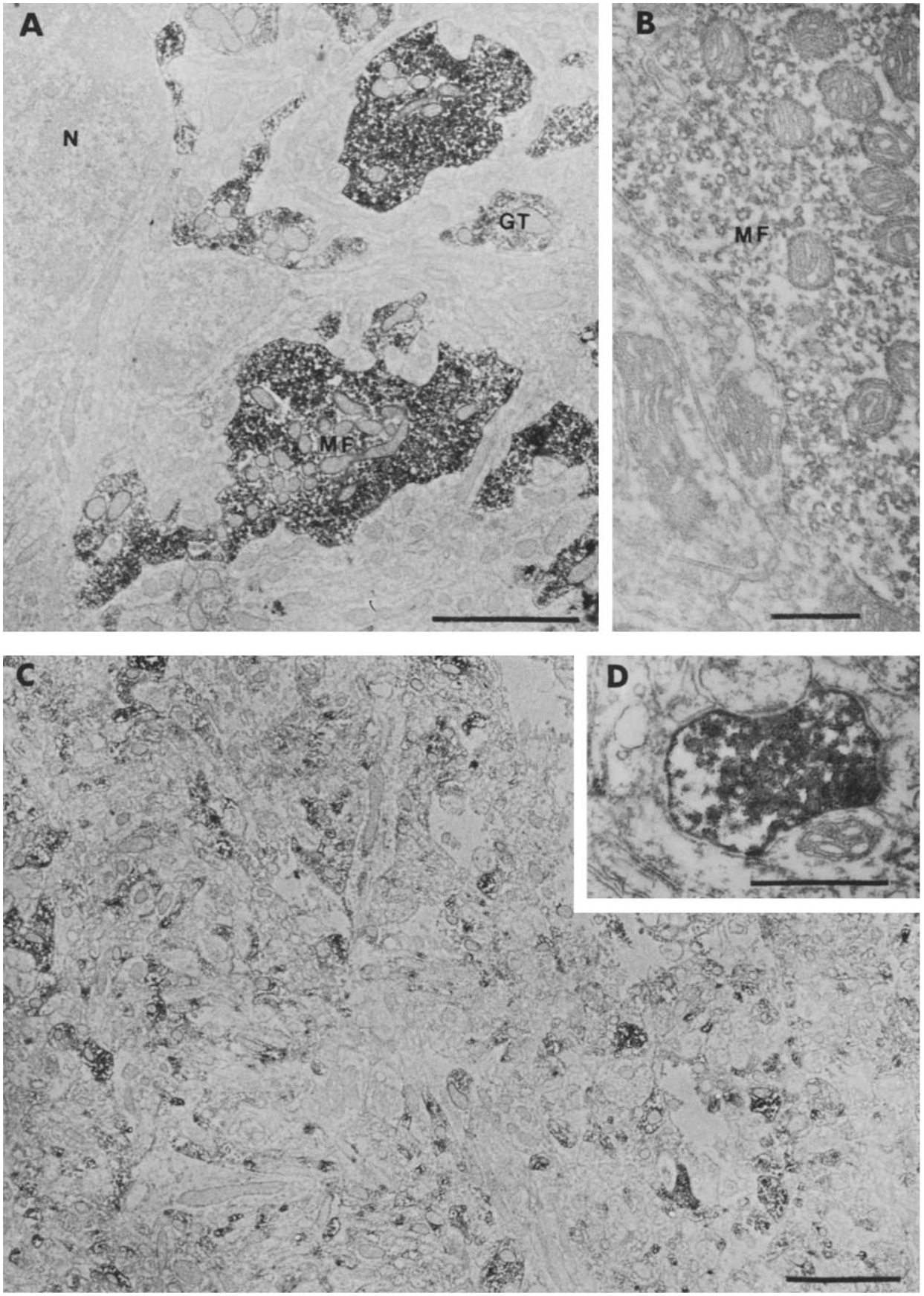

FIGURE 2 High resolution localization of serum 30 immunoreactivity in the cerebellum. These EM micrographs show sections of the granule cell layer in *A* and molecular layer in *C* with higher magnification micrographs of each layer in *B* and *D*. The peroxidase reaction product appears dark. In the granule cell layer *a*, both the large mossy fiber terminals (*MF*) and surrounding Golgi complex type II terminals (*GT*) have depositions of reaction product. In the molecular layer *C*, every terminal contains reaction product. Deposition of reaction product around vesicles is apparent in the higher power micrographs of a mossy fiber terminal in *B* and parallel fiber terminal in *D*. There may be some product on presynaptic terminal surface membranes, but it is never seen on postsynaptic membranes. Control experiments did not show reaction product. Bars: *A* and *C*, 2 μm; *B* and *D*, 0.5 μm.

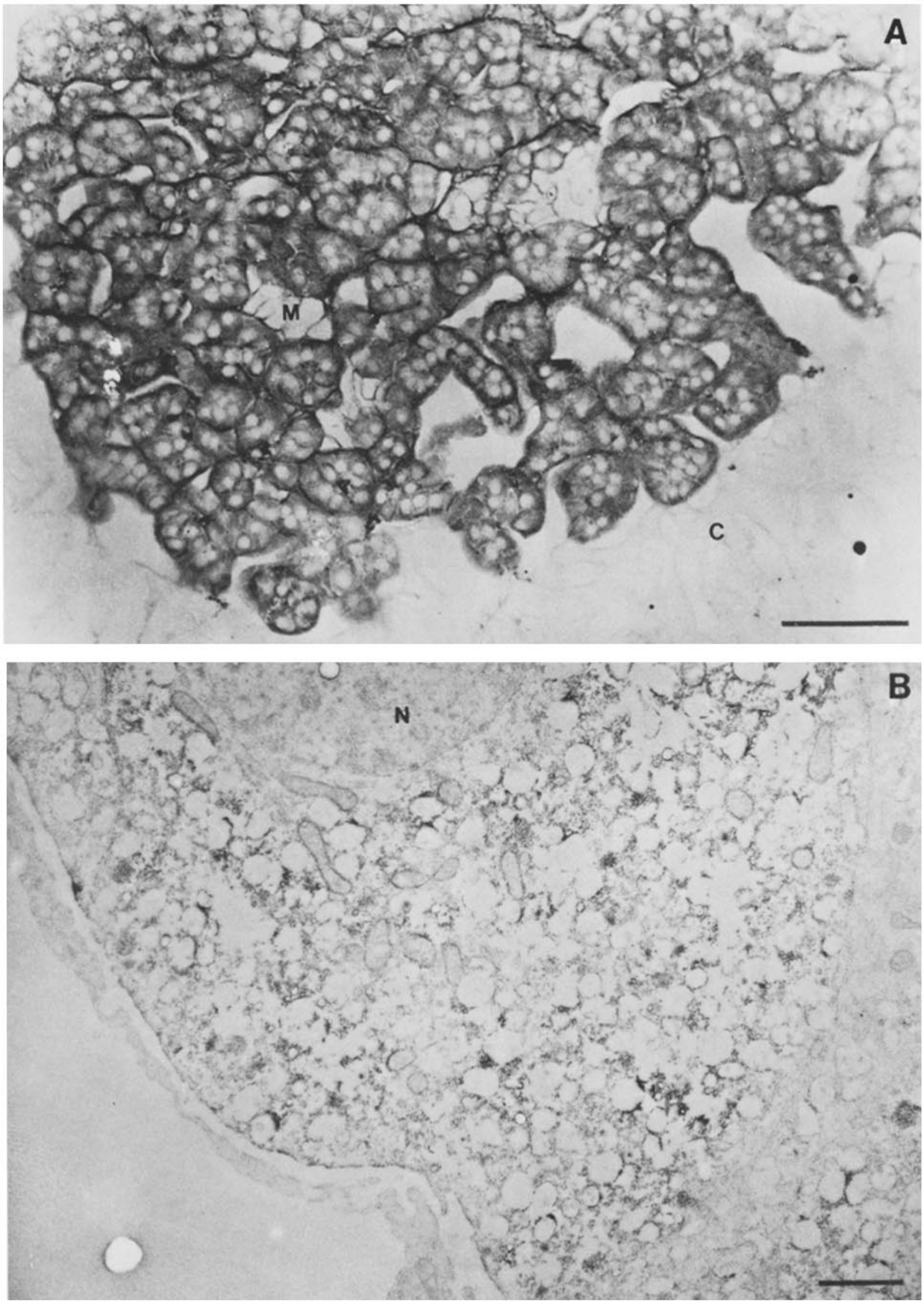

FIGURE 3 Localization of immunoreactivity in the adrenal medulla. In this figure are representative micrographs that show the localization of serum 30 immunoreactivity in the adrenal gland. In a low power micrograph (*A*) that includes portions of both the adrenal cortex (*C*) and medulla (*M*), the peroxidase reaction product is clearly found only in the medulla, where it is localized inside adrenal chromaffin cells. In an EM micrograph of chromaffin cells (*B*), a thin deposition of reaction product outlines the chromaffin granules and the inside surface of sinuisoidal portions of the plasmalemma. Control experiments did not show reaction product. Bars: *A*, 100 μm; *B*, 2 μm.

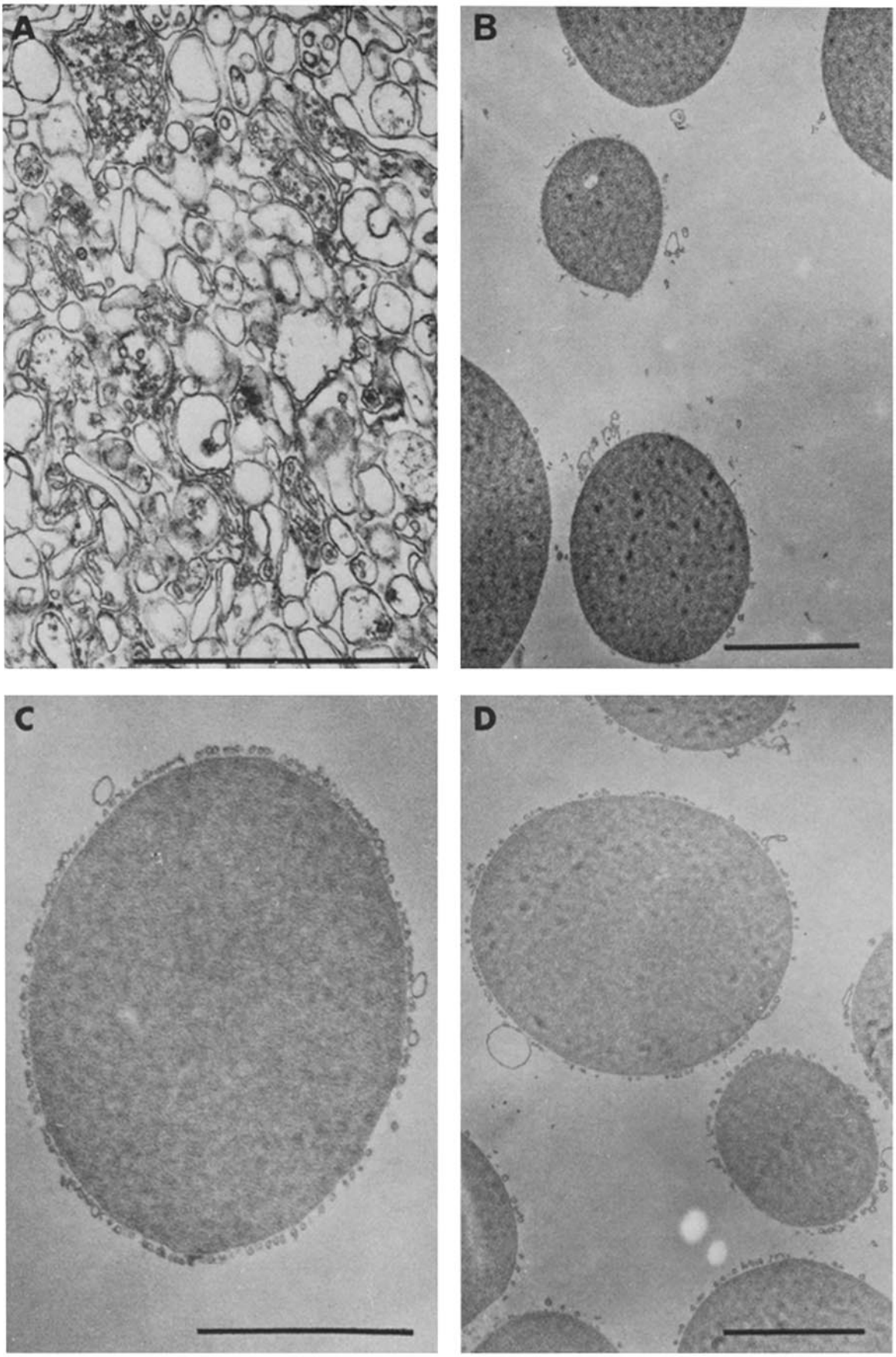

FIGURE 4 Membrane profiles immunoprecipitated on protein A-acrylamide beads. An EM micrograph of a sectioned crude brain homogenate is shown in *A*. The pellet was thoroughly mixed with an equal volume of buffer before fixation. An EM micrograph of a sectioned acrylamide bead with attached membrane profiles immunoprecipitated with normal mouse serum is shown in *B*. In *C* and *D* are profiles precipitated with serum 30. Procedures are described in Materials and Methods. Bars, 1 μm.

TABLE I

Selective Immunoprecipitation of Synaptic Vesicles with Serum 30

	Vesicles	Other profiles	Total profiles	Vesicles, other profiles
Crude homogenate	136	1,165	1,301	0.12
Profiles precipitated with serum 30	1,357	376	1,733	3.61

The profiles seen in EM micrographs of lysed crude brain homogenate (cf. Fig. 4 *a*) and a membrane fraction purified by immunoprecipitation (cf. Fig. 4 *c* and *d*) are tabulated. Only profiles that could be unambiguously identified as synaptic vesicles were counted in that category. These results are the average of two separate experiments that did not differ significantly from each other.

primarily by the rate at which they are released into the surrounding medium. Table III also has the results of a similar experiment in which the cells were grown for 5 d in [^{14}C]leucine and 1 h in [^{3}H]NE. In a homogenate, the [^{3}H]NE/[^{14}C]protein ratio was defined as 1. After immunoprecipitation of the vesicles with serum 30, this ratio was increased to >10. In Table II, we showed that 55% of the [^{3}H]NE was released from vesicles during the 4-h incubation period in our immunoprecipitation protocol, so the vesicle membranes and nonexchangeable contents were probably purified 20-fold. PC12 cells contain large numbers of vesicles, so a 20-fold purified preparation may be close to homogeneity, but this has not yet been proven. Since intact vesicles can be precipitated, the antigens must be exposed on the synaptic vesicle's outer surface, as was suggested by the distribution of peroxidase reaction product.

Nature of the Antigens and Antibodies

To determine the specificity of the antibodies and properties of the antigens bound by them, we denatured synaptic plasma membranes (29) in boiling SDS and separated the denatured proteins by size on polyacrylamide gels (33). After electrophoresis was completed, the proteins were fixed in the gels and antibody molecules were allowed to bind the proteins in the gel (5). In Fig. 5 (lanes *b* and *d*), it is evident that each antibody binds a single protein in these gels, which has a molecular weight of ~65,000. A mixture of the antibodies also binds only one band, Fig. 5*f*, so the antigenic determinants must be on proteins of similar molecular weight. In fact, the results in Fig. 6 show that each monoclonal antibody is able to block subsequent binding by an isotope-labeled preparation of the other antibody. This result implies that the two antibodies bind antigenic determinants on the same 65,000-dalton protein. The two antibodies are not identical, however. Serum 48 clearly binds with greater affinity than serum 30 (Fig. 6). In addition, they are members of different IgG allotype groups (data not shown). The protein is a minor component, not evident in the corresponding Coomassie Blue–stained gel strip (Fig. 5*a*, *c*, and *e*).

Further studies were done on the antigen using a quantitative radioimmune assay. The results in Table IV show that the antigen is associated almost exclusively with the particulate fraction from a brain homogenate. Less than 1% of the antigen is found in the soluble fraction. The antigen is not released from the particulate fraction by high or low salt, but is solubilized with Triton X-100. The results in Table IV also show that the antigen can be almost completely destroyed by incubation with trypsin. Therefore, the antigen has the properties of an integral membrane protein. These results are compatible with the observed association of the antigenic determinants with the outer surface of synaptic vesicles.

TABLE II

Immunoprecipitation of [^{3}H]NE from PC12 Homogenates

	Efficiency of immunoprecipitation of [^{3}H]NE		
	Total CPM sampled	CPM bound to millipore filter	CPM precipitated on beads
Cell homogenate: time 0	5,200	2,100	–
Cell homogenate: 4 h, 20°C	5,200	940	–
Cell homogenate + antibody 30: 4 h, 20°C	5,200	980	930

Efficiency of immunoprecipitation of [^{3}H]NE. 10^{6} PC12 cells were incubated with 1 μCi [^{3}H]NE for 80 min at 37°C (41). Cells were homogenized in 1 ml of growth medium with a glass-teflon homogenizer. Then large debris was removed by centrifugation at 10,000 *g* for 5 min. Aliquots were used to estimate the vesicular [^{3}H]NE content by millipore filtration (41). Preparations were incubated at room temperature and samples removed and adsorbed to millipore filters to determine the rate at which transmitter leaks out of these vesicles. Values are cited for 4 h—the time required to complete the immunoprecipitation on beads. Aliquots were used for immunoprecipitations: 300 μl aliquots were mixed with 40 μg of each antibody, incubated for 100 min at 2° C, and adsorbed to immobilized protein A.

TABLE III

Immunoprecipitation of [^{3}H]NE from a PC12 Homogenate Containing [^{14}C]Leucine-labeled Proteins

	[^{3}H]NE / [^{14}C]Leucine
Cell homogenate	1.0
Protein A–polyacrylamide bead	10.6

Immunoprecipitation of [^{3}H]NE from a PC12 homogenate containing [^{14}C]leucine-labeled proteins. PC12 cells were grown in the presence of [^{14}C]leucine for 5 d before a 60-min incubation in [^{3}H]NE. The ratio of [^{3}H]NE to [^{14}C]leucine was normalized to the value found in the homogenate which has been defined as 1.0.

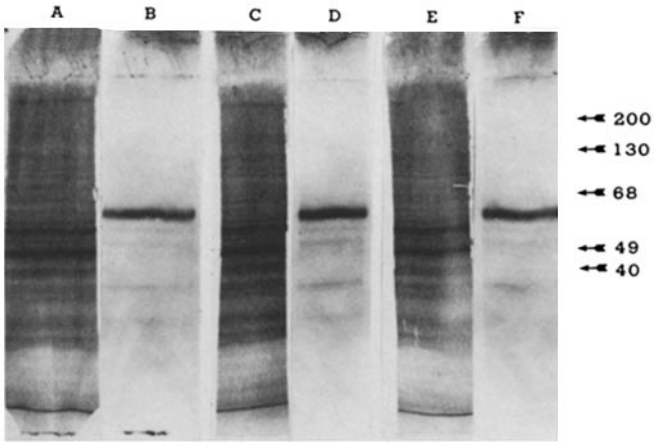

FIGURE 5 Identification of antigens. Synaptic plasma membranes were dissolved in SDS mercaptoethanol and fractionated in 10% polyacrylamide gels in a discontinuous buffer system (33). The antigens were identified by initial incubation with the monoclonal antibody, subsequent incubation with [^{125}I]-goat-anti-mouse-kappa chain affinity purified immunoglobulins (5). Lanes *A*, *C*, and *E* are Coomassie Blue–stained gel lanes; lane *B* is the autoradiograph of a gel treated with serum 30; lane *D* with serum 48; and lane *F* with both serum 30 and serum 48. The migration pattern of protein standards with molecular weights measured in thousands is shown on the right. Gels incubated with [^{125}I]-goat-anti-mouse-kappa antibody, but not either monoclonal antibody did not have detectable binding.

Widespread Distribution of the Antigenic Determinants in Neurosecretory Tissues

Because immunohistochemical procedures made it obvious that the 65,000-dalton protein was in many different classes of nerve terminals, we used an antigenic competition assay to measure its concentration in different parts of the nervous system, in non-neuronal secretory organs, and in nonsecretory tissues. The results in Table V show that the antigen is found in every part of the central or peripheral nervous system that was assayed. The antigen is found in cerebral and cerebellar cortices, midbrain, posterior pituitary, adrenal medulla, superior cervical ganglion, and a neuronal-like cell line—PC12. The concentration of antigen in two nonsecretory tissues—noninnervated regions of skeletal muscle and erythrocytes—was <0.4% of that found in the cerebral cortex, below the detection limits of this assay. Significantly higher levels of antigen were seen in non-neural secretory tissues, such as the liver, pancreas, and salivary gland. Since these concentrations are still only 1–5% of that found in the cerebral cortex, it seems possible that the antigen might be restricted to nerve terminals in these organs. Alternatively, the secretory vesicles in these organs may have very low amounts of the 65,000-dalton protein. Unfortunately, we cannot see such low levels of antigen with our immunohistochemical procedures. The anterior pituitary is a non-neuronal secretory tissue that contains more antigen than any other non-neural tissue, but only 10% of the activity seen in the posterior pituitary. The antigen is also found in two peptide-secreting cell lines, AtT 20 and GH3,

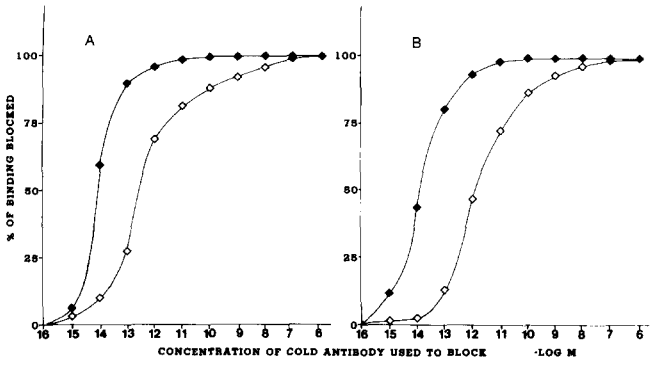

FIGURE 6 Competition for antigen by serums 30 (*B*) and 48 (*A*). 25 μl of 10 μg/ml rat brain synaptic plasma membranes were adsorbed to polyvinyl chloride microwells (32). After washing, 25 μl a dilution of ascites fluid was incubated 12 h with each well. The 25 μl of [^{125}I]-monoclonal antibody preparation was added for 4 h at 20°C. Wells were washed, dried, and counted. ◆, cold serum 48; ◇, cold serum 30.

TABLE V

Quantitative Measurement of Antigen in Different Tissues

Tissue and cell type homogenized	Quantitative measurement of antigen RSA*
	%
Tissue	
Extrajunctional diaphragm muscle (rabbit)	<0.3
Adrenal cortex (bovine)	<0.4
Pancreas (rabbit)	0.9
Liver (rabbit)	0.9
Salivary gland (rabbit)	5.4
Anterior pituitary (bovine)	11.0
Superior cervical ganglion (rabbit)	27.0
Adrenal medulla (bovine)	76.0
Posterior pituitary (bovine)	86.0
Cerebral cortex (rabbit)	75.0
Cerebellum (rabbit)	60.0
Superior colliculus (rabbit)	50.0
Whole brain (rat)	100.0
Cell type	
Blood cells (rabbit)	<0.2
HIT (hamster pancreatic islet)	<0.5
AtT20 (mouse anterior pituitary)	5.0
GH3 (rat anterior pituitary)	12.0
PC12 (rat adrenal phaeochromocytoma)	26.0
PC12 grown in nerve growth factor	38.0

Assay protocol is described in Materials and Methods. The amount of tissue protein needed to adsorb 50% of the binding was recorded from inhibition curves. The inverse values were normalized to rat brain which has been defined as 100% relative specific activity (RSA).

* % Relative Specific Activity $= \frac{\text{antigen units/mg tissue}}{\text{antigen units/mg adult rat brain}} \times 100.$

TABLE IV

Solubility Properties of the Vesicle Antigen

	Distribution of antigen					Antigen remaining after trypsinization
	(*a*) PBS	(*b*) 1 M NaCl	(*c*) 10 mM NaCl	(*d*) Triton X-100		(*e*) Protease
	%	%	%	%		%
Pellet	99.8	99.3	99.4	10.2	Pellet	<0.8
Supernate	0.2	0.7	0.6	89.8	Supernate	<50.0*
					Brain homogenate	<0.9

Adult rat cerebral and cerebellar cortices were removed, rinsed in 150 mM NaCl at 0°C, and homogenized at 0°C in 10 ml of 10 mM NaCl. 2 ml aliquots were diluted to 3 ml with final concentrations of 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and either 150 mM NaCl, 10 mM $NaPO_4$, pH 7.4 (*a*); 1 M NaCl (*b*); 10 mM NaCl (*c*); or 0.1% Triton X-100, 150 mM NaCl, 10 mM $NaPO_4$, pH 7.4 (*d*). These samples were incubated 15 min at 20°C before assay. Aliquots to be treated with protease, (*e*) were incubated 30 min at 37° C with 0.1 mg/ml diphenyl carbamyl chloride (DPCC)-trypsin (Sigma Chemical Co., St. Louis, Mo.), 2 mM $CaCl_2$, 150 mM NaCl, 10 mM $NaPO_4$, pH 7.4. Proteolysis was stopped with addition of 0.2 mg/ml soybean trypsin inhibitor, 1 mM PMSF, 2 mM EGTA, 2 mM EDTA. 200 μl of each sample were centrifuged at 100,000 *g* for 60 min in an airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellets were resuspended in 200 μl of the same solution and recentrifuged. Supernate from the two washes were pooled for assay. The final pellets were resuspended in 200 μl PBS. The total number of antigen units was determined for each sample with the quantitative radioimmune assay described in Materials and Methods. The samples which contained Trition X-100 were diluted 100-fold in PBS containing 5% calf serum to dilute the detergent which interferes with the plate assay. The supernates in *a*, *b*, *c*, and *d* were assayed directly or after equilibration in PBS by chromatography on a Biogel P2 column. Both procedures gave identical results. The total amount of antigen in each trypsinized sample was compared to the amount of antigen in samples from which membranes were adjusted to PBS immediately after homogenization. The values cited in *e* show the percent of antigen in the trypsinized samples relative to the amount of antigen in the untrypsinized samples.

* Represents limit of assay sensitvity.

TABLE VI

Quantitative Measurement of Antigen in Different Vertebrate Species

Brain homogenized	RSA
	%
Drosophila	<0.9
Chicken	77.0
Frog	86.0
Rabbit	87.0
Mouse	89.0
Rat	100.0
Cow	120.0
Shark	200.0

The procedure and definition of RSA are described in Materials and Methods and in the legend to Table IV.

which are derived from the anterior pituitary, further evidence that the antigen is in anterior pituitary cells. The distribution of antigen among the different cell types within the anterior pituitary has not yet been determined.

Evolutionary Conservation of the 65,000-dalton Protein

In Table VI are the results of an experiment in which the amount of antigen in different species was compared using the immune competition assay. While it was not detected in fruit flies, high levels were found in shark, chicken, frog, and every mammal examined. Clearly, the antigen has persisted through several hundred million years of evolutionary divergence.

In fact, the 65,000-dalton protein has retained its molecular weight and features of its conformation in these different species. The Burridge gel in Fig. 7 shows that the antibody binds the same molecular weight protein in brain homogenates of each species. This particular autoradiograph has been overexposed to show antibody binding to two minor bands at 40,000 and 50,000 daltons. While there are many possible explanations for these minor bands, it seems very likely to us that they are proteolytic fragments of the 65,000-dalton proteins. If so, the protease-sensitive sites have also been preserved in the different species, suggesting that the major structural features of the protein are highly conserved.

DISCUSSION

In this paper, we have identified a 65,000-dalton protein, greatly enriched in synaptic vesicles, that is widely distributed in the nervous system (Fig. 1; Table V). This protein is bound by two monoclonal antibodies (Figs. 5 and 6). Its molecular weight is based on its migratory behavior in SDS acrylamide gels in the presence of reducing agent (Fig. 5). The protein subunit could be part of a larger complex in situ. There are two arguments for the vesicular location of this protein: first, in PAP immunohistochemical studies the antigen is localized on the surface of synaptic vesicles (Figs. 2 and 3). Since solid-phase radioimmune assays on fractionated brain homogenates demonstrate that the antigen is in membranous, not soluble, fractions (Table IV), the immunohistochemical studies argue strongly that the antigen is found in synaptic vesicles, not in cytoplasmic material trapped during fixation. Secondly, when the antibodies are used to precipitate organelles from crude brain homogenates, they selectively precipitate synaptic vesicles (Fig. 4; Tables I, II, and III).

The determinants recognized by these antisera must be exposed on the outer surface of the vesicles, since the peroxidase reaction product is found outside the vesicle membrane profiles (Figs. 2 and 3), and the antisera can precipitate intact vesicles (Fig. 3; Table I) with neurotransmitter. These antibodies purify vesicles with a speed and efficiency comparable to more classical biochemical procedures (cf. reference 6; 22, 36, 37). They should be valuable reagents for purifying vesicles to greater homogeneity than hitherto possible and for rapid isolation of vesicular fractions.

Why might hybridoma cell lines secreting antivesicle sera be isolated from a mouse immunized with synaptic junctional complexes? Probably this vesicle protein was in our immunizing material, since the antibodies bind to detergent-extracted membranes (Table IV). We do not know whether the bulk of the antigen is actually present in the junctions or whether synaptic vesicle-derived material contaminates Triton-extracted preparations.

Solid-phase radioimmune competition assays (Table VI) and Burridge gels (Fig. 6) make it clear that the 65,000-dalton protein with associated antigenic determinants is conserved through the vertebrate evolutionary tree. The quantitative immune competition assays (Table V) show that the determinants are present in every tested neuronal or neurosecretory tissue at comparable concentrations. In the cerebellum, the antigen is clearly in both excitatory (mossy fiber, parallel fiber) and inhibitory terminals (Golgi complex type II) (Figs. 1 and 2). The transmitters have not been definitively identified at these cerebellar synapses, although glutamate is thought to be the transmitter in parallel fibers (cf. reference 26). Since every terminal has antigen in many of our low power EM micro-

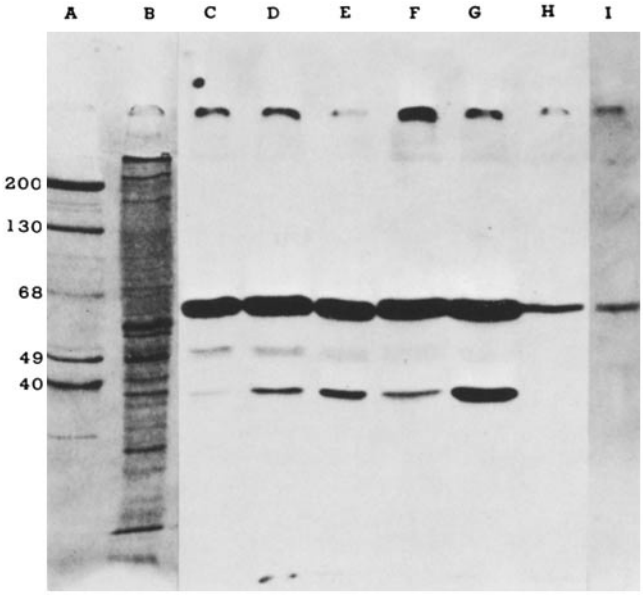

FIGURE 7 Identification of antigen in different vertebrate species. A sample of a whole brain homogenate was dissolved in SDS mercaptoethanol and processed as described in Fig. 5 and Materials and Methods. Gel lanes containing protein standards and rat brain, both stained with Coomassie Blue are shown at the left (lanes *A* and *B*). The molecular weights of the protein standards in thousands are indicated. The different species used as sources for the brain homogenates were rat (*C*), mouse (*D*), rabbit (*E*), cow (*F*), chicken (*G*), frog (*H*), and shark (*I*). Gels incubated with only the second antibody did not have detectable binding.

graphs (Fig. 2), it seems likely that the other terminal types in the cerebellum may contain this 65,000-dalton protein also. At the level of resolution provided by the light microscope, the antigen appears to be present in every visible synaptic layer of those portions of the nervous system that have been examined (Fig. 1), including cerebellum, retina, spinal cord, hippocampus, sympathetic ganglion, and pituitary. The antigen is present in putative glutaminergic and GABAnergic (gamma aminobutyric acid, GABA) terminals (cerebellar parallel fibers and Golgi complex type II endings), catecholaminergic tissue (adrenal, PC12), serotonergic tissue (pineal), and cholinergic cells or endings (PC12, presynaptic terminals in sympathetic ganglion). It is also found in a variety of peptidergic cells or terminals (the LHRH terminals in the frog sympathetic ganglion [Lily Jan and Yuh Nung Jan, personal communication], growth hormone-secreting GH3 cells, ACTH and endorphin releasing AtT20 cells, posterior and anterior pituitary [Table V]). We are left with the conclusion that this protein is very widely distributed in synaptic vesicles and may be present in every type of neuronal terminal.

Most vesicle-associated antigens that have been characterized have much narrower distributions than the 65,000-dalton protein described in this paper. The neurotransmitters and peptides are restricted to particular cell types (20, cf. reference 38). The vesicle-associated enzyme, dopamine-β-hydroxylase, is found only in cells that synthesize norepinephrine and epinephrine (cf. reference 45). The antiserum to elasmobranch cholinergic vesicles binds some adrenergic and peptidergic in addition to cholinergic terminals (21). Binding in the cerebellum and hippocampus is concentrated in presumptive cholinergic terminals (where high AChesterase is detected), a much more restrictive distribution than seen with the 65,000-dalton protein (21). It is also not certain whether this antiserum recognizes the same antigens in each terminal and whether these are localized in the membrane or interior of the vesicles.

The most likely vesicle-associated antigens to have distributions as broad as the 65,000-dalton protein are protein I and certain cytoskeleton-associated proteins. For example, Jokusch et al. (28) presented evidence that alpha-actinin was found in both adrenal chromaffin granules and platelet granules. A complete set of immunological controls and specificity tests was not included in this paper. If the result is valid, though, alpha-actinin would seem likely to be very widely distributed in vesicle preparations. Tubulin and actin have also been reported to be in preparations of cerebral synaptic vesicles (53) and adrenal chromaffin granules (37), respectively. If these are not contaminants, they would also be likely to have wide distributions. Since all these proteins are found in many other cellular structures, though, they are not very useful in studies of vesicles *per se*. In contrast, protein I, a major natural substrate for cAMP and Ca^{++}-activated protein kinases, is found only in neurons where it is associated with synaptic vesicles and postsynaptic densities (3). It has been identified in a wide variety of terminals in the CNS and autonomic nervous system (10). While preliminary studies failed to find it in several classes of synapses, including cholinergic terminals in skeletal muscle and pancreas, outer plexiform layer of the retina, parallel fiber terminals in the cerebellum, and many terminals in hippocampus (3, 10), current experiments indicate that protein I is present in many of those terminals at concentrations too low to be detected with the original procedure (P. Greengard, personal communication). Thus, protein I may be universally distributed in the nervous system, and its specific distribution pattern makes it likely to modulate vesicle function.

It is not known whether the 65,000-dalton protein corresponds to any of the proteins identified in purified vesicle fractions. Since its molecular weight is significantly less than that of protein I (80,000) or alpha-actinin (95,000), it can not be either of those proteins. In confirmation of this, we failed to detect binding with monoclonal antibodies, 30 and 48, to a purified preparation of protein I, generously donated by Dr. Greengard. Fractionation of the proteins associated with adrenal chromaffin granule membranes reveals minor proteins of approximately the right molecular weight (23, 37), but no experiments were done to see which minor proteins are purified with chromaffin granules and which are contaminants. Similarly, the proteins from brain synaptic vesicles have been fractionated on one- and two-dimensional acrylamide gels, revealing a plethora of proteins, some in the right molecular weight range (cf. reference 1), but careful studies to determine which are vesicular and which are contaminants have not been done. In their elegant study on purified cholinergic vesicles from the elasmobranch electroplax, Wagner and Kelly (50) found only eight major proteins in the vesicle, six of which copurified with vesicles. Intriguingly, one of these vesicle specific proteins has a molecular weight close to 65,000 and has a domain on the vesicle's outer surface.

The outer surface of the synaptic vesicle is likely to contain determinants that sort vesicular membrane during biogenesis, direct transport to the nerve terminal, modulate fusion with the plasmalemma, and permit selective retrieval after exocytosis (cf. references 4, 11, 13). Many classes of vesicle membranes also contain proteins responsible for maintaining electrogenic pumps and uptake of specific transmitter substances (cf. reference 30). Since the 65,000-dalton protein is found in many different types of terminals (Table V; Figs. 2 and 3), it is not likely to be associated with any transmitter-specific activity. The evolutionary conservation of its antigenic determinants suggests an important function, discovery of which may be difficult at this primitive stage of in vitro reconstitution of membrane biogenesis, sorting, and fusion (cf. reference 12). The monoclonal antibodies, however, demonstrate that immunological purification of organelles is an effective addition to the limited number of available biochemical techniques. They also show that many different types of neurosecretory vesicles share at least one common vesicle-specific protein, a relationship whose basis is probably in a common function.

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