

# Protein p38: An Integral Membrane Protein Specific for Small Vesicles of Neurons and Neuroendocrine Cells

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**Abstract.** An intrinsic membrane protein of brain synaptic vesicles with  $M_r$  38,000 (p38, synaptophysin) has recently been partially characterized (Jahn, R., W. Schiebler, C. Ouimet, and P. Greengard, 1985, *Proc. Natl. Acad. Sci. USA*, 83:4137–4141; Wiedenmann, B., and W. W. Franke, 1985, *Cell*, 41:1017–1028). We have now studied the presence of p38 in a variety of tissues by light and electron microscopy immunocytochemistry and by immunochemistry. Our results indicate that, within the nervous system, p38, like the neuron-specific phosphoprotein synapsin I, is present in virtually all nerve terminals and is selectively associated with small synaptic vesicles (SSVs). No p38 was detectable on large dense-core vesicles (LDCVs). p38 and synapsin I were found to be present in similar concentrations throughout the brain. Outside the nervous system, p38 was found in a variety of neuroendocrine cells, but not in any other cell type. In neu-

roendocrine cells p38 was localized on a pleiomorphic population of small, smooth-surfaced vesicles, which were interspersed among secretory granules and concentrated in the Golgi area, but not on the secretory granules themselves. Immunoblot analysis of endocrine tissues and cell lines revealed a band with a mobility slightly different from that of neuronal p38. This difference was attributable to a difference in glycosylation.

The finding that p38, like synapsin I, is a component of SSVs of virtually all neurons, but not of LDCVs, supports the idea that SSVs and LDCVs are organelles of two distinct pathways for regulated neuronal secretion. In addition, our results indicate the presence in a variety of neuroendocrine cells of an endomembrane system, which is related to SSVs of neurons but is distinct from secretory granules.

**R**EGULATED vesicular secretion of neurotransmitter molecules from nerve endings and regulated vesicular secretion from non-neuronal cells have a number of features in common (23, 31). However, nerve ending secretion also shows some specific features. At least two types of secretory organelles, small synaptic vesicles (SSVs)<sup>1</sup> and large dense-core vesicles (LDCVs), coexist in some and possibly all nerve endings, where they appear to be involved in two distinct secretory pathways (24, 27, 32, 38). SSVs (40–60-nm diam) appear to be storage sites for only classical neurotransmitters, i.e., small non-peptide molecules (27). These vesicles undergo a local exo-endocytotic recycling in nerve terminals. At each cycle they can be reloaded with neurotransmitter because the machinery necessary to synthesize and load classical neurotransmitters

into vesicles is present in the axon terminal and a direct involvement of the cell body is not required (6, 38). SSVs are regarded as neuron-specific organelles with no equivalent in non-neuronal cells (32). In contrast, LDCVs, i.e., larger vesicles (>60-nm diam) with an electron-dense core, appear to be storage sites for peptide neurotransmitters (24, 27). The nerve terminal is dependent upon a constant supply of preassembled LDCVs from the perikaryon, since peptide neurotransmitters, or their pro-peptide precursors, can only be synthesized and loaded into vesicles in perikarya. LDCVs can be considered equivalent to secretory granules of endocrine cells, which they resemble in morphology, content, and mechanism of assembly (32).

Some recent studies have suggested that the release from SSVs and LDCVs may be, at least partially, independently regulated (1, 27). Furthermore, it has been found that the phosphoprotein synapsin I (8–10, 20, 41, 44) is preferentially, and perhaps exclusively, associated with the surface of SSVs (32). Synapsin I is a neuron-specific protein that is

1. *Abbreviations used in this paper:* control-IgGs, nonimmune rabbit IgGs; p38-IgGs, affinity-purified rabbit serum antibodies directed against p38; syn I-IgGs, affinity-purified rabbit serum antibodies directed against synapsin I; LDCVs, large dense-core vesicles; SSVs, small synaptic vesicles.

highly concentrated in virtually all nerve endings (8, 10), where it is tightly bound to the cytoplasmic surface of the vesicles (10, 20).

To further understand the structural and functional relationship between SSVs and LDCVs, and the relationship between these vesicles and the secretory vesicles of non-neuronal cells, it is important to characterize and compare the molecular characteristics of intrinsic membrane components of SSVs and LDCVs. Recently we have characterized an intrinsic membrane protein of  $M_r$  38,000 (p38) present in a highly purified synaptic vesicle fraction (22). This protein is probably identical to a protein with the same apparent molecular weight identified in brain coated vesicles (36, 49) and in presynaptic vesicles (47, referred to as synaptophysin in that study). It is an acidic glycoprotein, with a large cytoplasmic domain, which appears to have a widespread distribution at synapses (22, 37, 47).

In the present study we have investigated the distribution and subcellular localization of p38 in a variety of tissues. We report that p38, like synapsin I, is present in all nerve endings, where it is selectively localized in the membranes of SSVs, with little or none being present in the membranes of LDCVs. We show further that p38, at variance with synapsin I, has a widespread distribution in a variety of neuroendocrine cells, and that in these cells p38 is localized on small pleiomorphic vesicles with clear content, but not on secretory granules. Preliminary accounts of this work have been presented previously (33).

## Materials and Methods

### Antibodies

Monoclonal antibodies (IgG clones C 7.2 and C 7.3) and rabbit serum antibodies against p38 were generated as described previously (22). Monoclonal antibodies were purified from ascites fluid using the method of Bruck et al. (4). Serum antibodies directed against p38 (p38-IgGs) were affinity purified using a denatured membrane fraction from rat brain as immunoadsorbent by the following procedure. (All steps were carried out at 4°C). Starting with 15 rat brains, a P2-fraction was prepared as described (20). The P2 pellet was resuspended in 15 ml of 0.1 M glycine/HCl pH 2.4, homogenized vigorously using a glass-Teflon homogenizer, and further dispersed by squeezing it through a 30-gauge needle. After 20 min of stirring, the sample was centrifuged at 5,000 g for 15 min. The pellet was washed three times in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na-phosphate, pH 7.2), resuspended in 10 ml of anti-p38 serum, and vigorously homogenized. After incubation under rotation for 2 h, the mixture was centrifuged at 15,000 g for 15 min and the pellet was washed five times with PBS. The final pellet was resuspended as above in 10 ml of 0.1 M glycine/HCl, pH 2.4 and the mixture incubated for 30 min under rotation to allow dissociation of the antibody. The solubilized antibody was recovered by centrifugation at 15,000 g for 15 min. The pellet was re-extracted once by the same procedure. The combined supernatants were immediately neutralized with 1 M Tris-base, dialyzed against PBS, and concentrated using an Amicon ultrafiltration apparatus (Amicon Corp., Danvers, MA). This is the fraction referred to as "p38-IgGs." Electrophoretic analysis revealed mostly IgG and some IgM, with no detectable contaminants. A typical yield was 0.8 mg of protein.

Both monoclonal and polyclonal antibodies recognized almost exclusively one band of  $M_r$  ~38,000 when tested by immunoblot analysis against proteins of total homogenates of rat and bovine tissues, which had been separated by SDS gels under reducing conditions (22; see also Fig. 9, top). In addition to this band, only a small immunoreactive band of  $M_r$  76,000 was sometime seen (Fig. 9, top). This corresponded to a dimeric form of p38. Most epitopes recognized by these antibodies are located on the cytoplasmic portion of the molecule (22).

Affinity-purified rabbit polyclonal IgGs directed against synapsin I (syn I-IgGs) were prepared and characterized as previously described (8, 10).

Affinity-purified antibodies directed against secretogranin II (40) were a kind gift of A. Zanini (Milano). Nonimmune rabbit IgGs (control IgGs) and rhodamine-conjugated goat anti-rabbit IgGs were from Cooper Biochemical, Inc. (Malvern, PA).

### Purification of p38

p38 to be used as a standard for quantitative immunoassay was purified by affinity chromatography after detergent extraction of a rat brain membrane fraction. 15 male Sprague-Dawley rats (200–250 g) were killed by decapitation. The forebrains were quickly removed, freed from blood vessels, and homogenized in 150 ml of 0.32 M ice-cold sucrose containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO) and 2 µg/ml pepstatin (Chemicon, El Segundo, CA) using a glass-Teflon homogenizer. The homogenate was centrifuged at 800 g for 10 min. The resulting low-speed supernatant was spun at 100,000 g for 90 min. The resulting high-speed pellet was homogenized in 100 ml of 150 mM NaCl, 10 mM Na-phosphate pH 7.2, 0.1 mM PMSF, and 2 µg/ml pepstatin containing 1% (vol/vol) Triton X-100. After stirring on ice for 60 min, the extract was spun again for 90 min at 100,000 g. The resulting supernatant (Triton extract) was used for affinity chromatography. Occasionally the pellet (Triton pellet) was re-extracted once, providing a somewhat higher yield.

An affinity resin was prepared by coupling 25 mg of a mixture of purified monoclonal antibodies C7.2 and C7.3 to 3.5 ml of CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The Triton extract was loaded, and the column was washed with 10 ml of PBS containing 1% (vol/vol) Triton X-100, followed by 10 washes using alternatively 10 ml of high pH buffer (0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl pH 8.3, 0.1% [vol/vol] Triton X-100) and 10 ml of low pH buffer (0.1 M Na-acetate pH 5.5, 0.5 M NaCl, 0.1% [vol/vol] Triton X-100). The column was finally washed with 10 ml of PBS containing 0.1% (vol/vol) Triton X-100 and eluted with 20 ml of 3.5 M NaSCN containing 0.1% (vol/vol) Triton X-100. The eluate was dialyzed extensively against PBS and finally concentrated to 2–3 ml using an Amicon ultrafiltration apparatus. The final product did not contain any significant contaminant as judged by SDS PAGE. p38 was purified 313-fold over the homogenate, with virtually all of the purification being achieved by the affinity chromatography step. p38 was enriched from PC12 homogenate (kindly provided to us by Dr. R. Nichols, the Rockefeller University) using the same procedure as used for the brain protein.

### Light Microscopy Immunocytochemistry

**Preparation of Tissue Sections.** Sprague Dawley albino rats, 175–250 g, were anesthetized and fixed by transcardial perfusion as described (8). The fixative used was 4% formaldehyde (freshly prepared from paraformaldehyde) or 3% formaldehyde/0.25% glutaraldehyde. At the end of the perfusion, brains and other tissues of interest were removed, cut into small blocks, and immersed in the same fixative for an additional 3 h. After a rinse in PBS (several hours), some blocks were dehydrated in graded ethanols and propylene oxide and embedded in Epon for plastic sectioning. Other blocks were infiltrated with either 0.5 or 2.3 M sucrose and subsequently frozen in isopentane chilled with liquid nitrogen. Tissue infiltrated with the lower sucrose concentration was cut in a Reichert-Jung (Vienna, Austria) cryostat (10-µm thick frozen sections), the other tissue in a Reichert-Jung FC4/Ultracut (1-µm thick frozen sections). Preparation of 1-µm thick plastic sections and plastic removal were performed as described (8).

**Immunostaining and Photography.** Immunostaining of sections was performed by an indirect immuno-rhodamine procedure as described (8), with the exception that affinity-purified serum antibodies rather than whole sera were used in the primary labeling step. In all cases antibodies were used at the final concentration of 0.1 mg/ml. At the end of the immunostaining, glass slides were mounted with 95% (vol/vol) glycerol in 120 mM sodium phosphate buffer and observed with a Zeiss photomicroscope III equipped with epifluorescence and planapo objectives. Pictures were taken with technical pan 2415 film, which was developed with undiluted D 19. Films and chemicals were from Kodak.

### Electron Microscopy Immunocytochemistry

**Agarose-embedded Subcellular Particles.** Fresh bovine brains and adrenals were obtained from the Milano public slaughterhouse. Within 15–20 min from the time of death, brains and adrenal glands were removed from the animals, and the tissues of interest were dissected out. Tissues were homogenized by several manual strokes in 6 vol of ice cold 0.25 M sucrose/5 mM sodium phosphate buffer in a glass-Teflon homogenizer. The hypotaha-

lamic homogenate was then divided into two aliquots that were fixed separately. One aliquot was fixed by addition of 1 vol of twofold concentrated fixative in 0.25 M sucrose/5 mM sodium phosphate buffer (nonlytic fixation). The other aliquot was fixed by addition of a large amount (more than 15 vol) of onefold concentrated fixative in 5 mM phosphate buffer (lytic fixation). For a discussion of lytic versus nonlytic fixation, see reference 10 and Results. The homogenate of the adrenal medulla was fixed by addition of 1 vol of twofold concentrated fixative in 0.25 M sucrose/5 mM sodium phosphate buffer. In all cases the final fixative was 3% formaldehyde (freshly prepared from paraformaldehyde)/0.25% glutaraldehyde.

After a 30-min fixation on ice, subcellular particles of the homogenate were pelleted by centrifugation (13,000 *g* for 45 min). The compact pellet was scraped with a spatula from the bottom of the tube, resuspended in a small volume of 120 mM sodium phosphate buffer, and gently rehomogenized in a small glass-Teflon homogenizer. The thick suspension obtained was mixed with warm liquid agarose to prepare blocks of agarose-embedded particles (10). Immunostaining of agarose blocks was performed as described, using affinity-purified antibodies at the first labeling step and protein A-colloidal gold at the second labeling step. Preparation of gold sols and their conjugation to protein A was carried out according to Slot and Geuze (42). After the second labeling step, agarose blocks were further fixed with 1% glutaraldehyde in 120 mM sodium phosphate buffer, then postfixed in 1% OsO<sub>4</sub>, dehydrated, and embedded in Epon as described (10). Thin sections were prepared with an Ultracut Microtome (Reichert-Jung) and examined in Phillips 301 and 400 electron microscopes.

**Ultra-thin Frozen Sections.** Preparation and immunolabeling of ultra-thin frozen sections of rat anterior pituitary were performed essentially as described (18). Briefly, the tissue was fixed by perfusion as for light microscopy immunohistochemistry, but using 4% formaldehyde/0.05% glutaraldehyde as the fixative. Before sectioning in a Reichert-Jung FC4/Ultracut, small tissue blocks were infiltrated in 2.3 M sucrose and frozen in liquid nitrogen. Immunolabeling was performed as described (18) with the exception that (a) step 4 was preceded by a 20-min incubation in a solution containing 15% fetal calf serum/0.45 M NaCl/0.02 M sodium phosphate buffer (buffer A), (b) IgGs (final dilution 0.1 mg/ml) and protein A-gold were diluted in buffer A, and (c) after step 7, grids were floated on a 2% solution of glutaraldehyde in PBS and then rinsed by floating them on PBS alone. Contrasting and plastic embedding of the labeled grids were then performed by processing them through the following steps: (a) rinse with 0.1 M acetate-veronal (AV) buffer pH 7.4 (twice for 2 min each); (b) postfix with 1% OsO<sub>4</sub> in AV buffer (15 min); (c) rinse with AV buffer (twice for 2 min each); (d) counterstain with 0.5% uranyl acetate in AV buffer, pH 6.5 (15 min); (e) wash with distilled water (three times for 2 min each). All these steps were performed by floating the grids on a drop of solution. Grids were subsequently dehydrated and plastic embedded by transferring them first through a series of tubes containing ethanol solutions of increasing concentrations (from 50 to 100%), and then through a series of three tubes containing a solution of LR white. Excess LR white was then removed by sandwiching grids between two small layers of hard Whatman No. 50 (Maidstone, England) filter paper which were gently pressed against each other. Finally grids were left in a 60°C oven overnight to allow the embedding resin to polymerize.

### Other Techniques

Quantitation of synapsin I and p38 in total homogenates was performed using a dot-immunobinding assay (21). p38 prepared from rat brain and from PC12 cells as described above was chemically deglycosylated by trifluoromethanesulfonic acid using the method of Edge et al. (14) as modified by J. David Castle (Yale University) and published by Bartles et al. (2). SDS PAGE was carried out according to Laemmli (25) using a slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) and 9–13% linear gradient gels 1.5-mm thick. Blotting was performed according to Towbin et al. (43). Immunostain of the blots by a peroxidase procedure was performed as described (22). Protein was determined by a modification (28) of the method of Lowry et al. (26). Primary cultures of bovine adrenal chromaffin cells were a kind gift of R. A. Nicholas and J. Haycock (The Rockefeller University).

## Results

### Distribution of p38 in the Nervous System and Comparison with the Distribution of Synapsin I

Immunofluorescence staining of frozen (10- $\mu$ m thick) and

plastic (1- $\mu$ m thick) sections of a variety of brain areas with p38-IgGs produced a punctate pattern of staining in all nerve terminal-containing regions, leaving all regions of the white matter unstained. The morphological distribution of p38 was very similar to that of synapsin I in practically all nerve terminal-containing regions of the brain examined. A comparison of the distribution of the two antigens in two brain regions is shown in Fig. 1.

Fig. 1, *a* and *b* shows a pair of sections of the cerebellar cortex. In this brain region both p38 and synapsin I immunoreactivities occur in the form of small dots in the molecular layer and of large irregular islands in the granule cell layer. This is precisely the distribution expected for nerve terminals (8). Individual dots in the molecular layer correspond to single axon terminals, primarily parallel fiber terminals, by far the predominant type of terminal in this layer. Islands of immunoreactivity in the granule cell layer represent mossy fiber terminals and satellite nerve terminals of the cerebellar glomeruli.

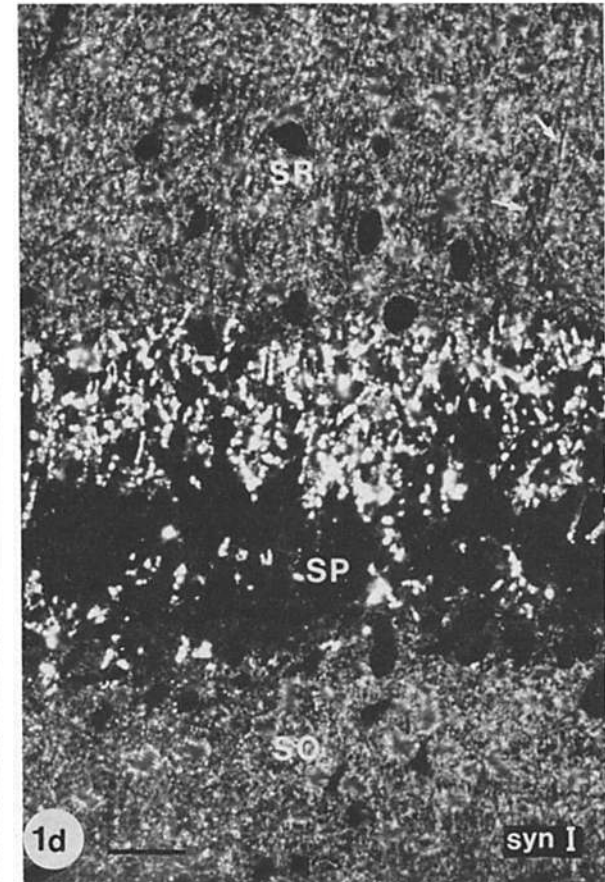
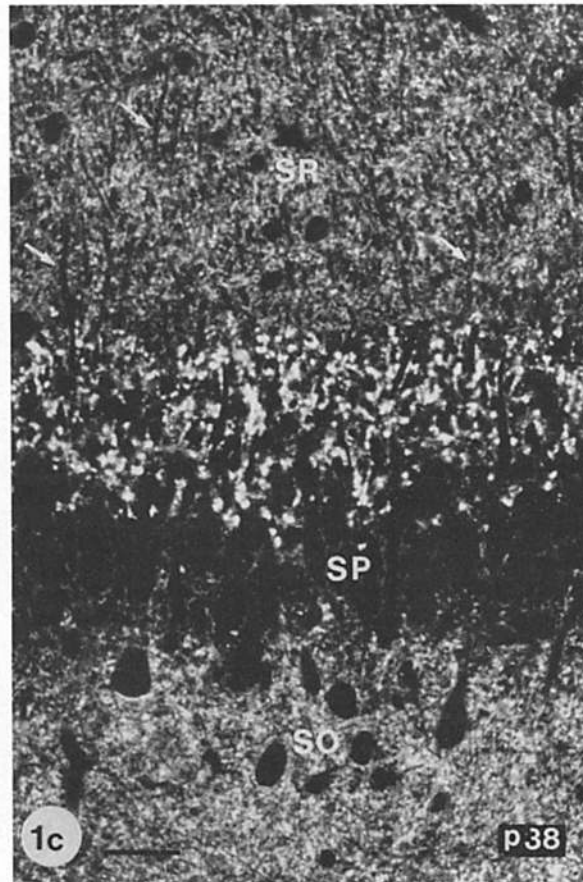
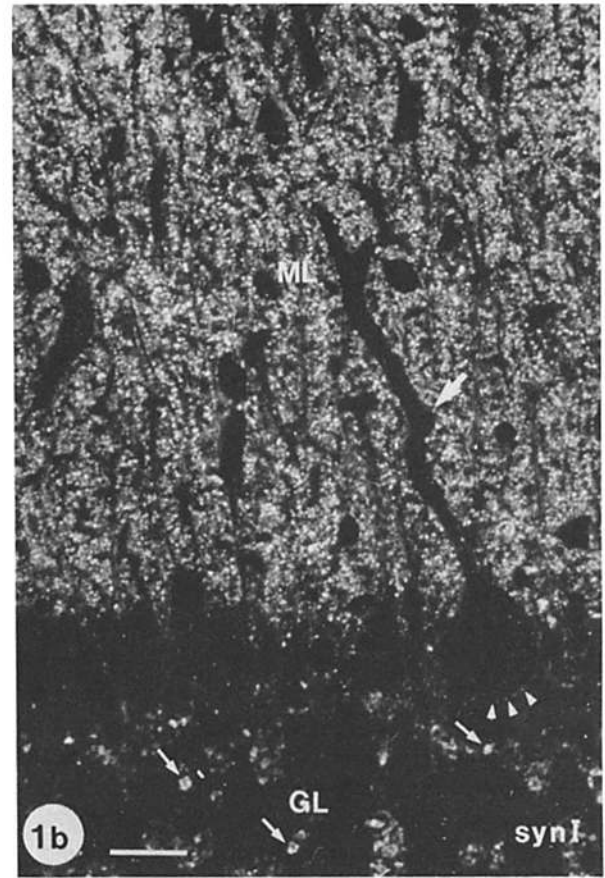
Fig. 1, *c* and *d* shows a pair of sections of the CA3-CA4 region of the hippocampus. In this hippocampal region, a predominant population of very small fluorescent dots is seen in the stratum oriens and in the top portions of the stratum radiatum. These dots represent primarily small terminals around distal apical dendrites (in the stratum radiatum) and around basal dendrites (in the stratum oriens) of pyramidal cells. A population of large, brightly fluorescent dots, most of which are located in the bottom part of the stratum radiatum, corresponds to the very large mossy fiber terminals which establish multiple synaptic contacts with spines of proximal portions of pyramidal cell apical dendrites (8).

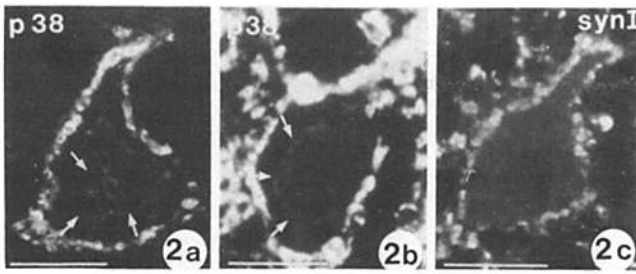
The similarity of the p38 and synapsin I staining patterns described above for two brain regions was found in all axon terminal-containing regions of the central nervous system studied with the exception of the retina (see Discussion). Similar results were obtained in the peripheral nervous system, where the presence of p38 was demonstrated by immunofluorescence in autonomic ganglia and in all innervated tissues examined. These included the striated muscle, the vas deferens, various regions of the gastrointestinal system, and a variety of exocrine and endocrine glands (see for example Fig. 6 *c* and not shown). The distribution of p38 was consistent with its presence at all SSV-containing regions, and, in all cases, the p38 staining pattern on neuronal processes was very similar to that of synapsin I.

A difference in the distribution of the two antigens was only observed in the perikaryal region of neurons, where both antigens were present at low concentration (Fig. 2). p38-IgGs stained elongated, irregularly shaped, particles, which formed a network around the nucleus. The morphological distribution of these particles inside the cell corresponded to that of the Golgi complex (11). Such a network of particles was consistently observed in all neurons, even though the intensity of the staining varied from one cell type to another. Examples of such perikaryal networks can be seen in the two neurons shown in Fig. 2, *a* and *b*. In contrast, syn I-IgGs produced a faint diffuse staining throughout the cytoplasm of perikarya and major dendrites (Fig. 2 *c*).

None of the staining patterns described was seen when control IgGs were substituted for p38- or synI-IgGs (not shown).

Consistent with the morphological results, the ratio be-





**Figure 2.** Comparison of the distribution of p38 (*a* and *b*) and synapsin I (*c*) immunoreactivities in the perikaryal regions of neurons. 1- $\mu$ m thick plastic sections stained by immunofluorescence. (*a* and *b*) The figures show the perikarya of two brain stem neurons outlined by immunoreactive terminals of axosomatic synapses. Inside the cytoplasm a network of faintly labeled perinuclear particles (*arrows*) can be seen (the nucleus of the neuron shown in *a* is out of the plane of section). These have the distribution expected for elements of the Golgi complex. (*c*) Neuron of the deep cerebellar nuclei. A very weak diffuse fluorescence is visible throughout the cytoplasm but not in the nucleus. Bars, 40  $\mu$ m.

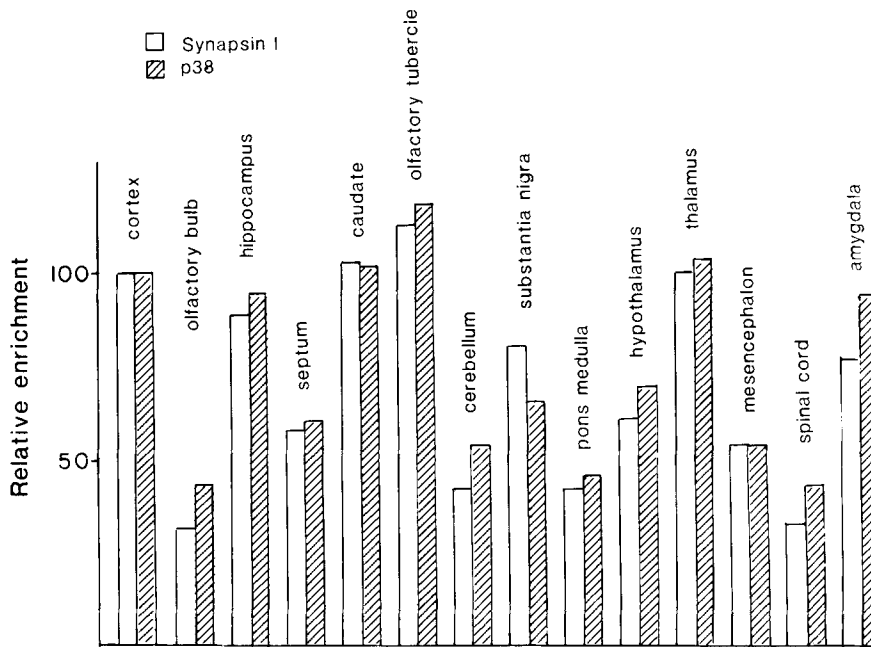
tween p38 content and synapsin I content, as assessed by a quantitative dot-immunobinding assay, was found to be quite constant in all brain regions examined (Fig. 3). The absolute amount of p38 in brain tissue was determined using purified p38 as a standard. In cerebral cortex homogenate, the

amount of p38 was 3.2  $\mu$ g/mg total protein, which is almost identical to the value (3.8  $\mu$ g/mg total protein) for synapsin I reported earlier (15). Since p38 seems to be a dimer cross-linked with S-S bonds in its native form (37; our unpublished observations) yielding a native molecular mass of 76,000 D, the stoichiometric ratio of synapsin I to p38 is close to 1.

### Subcellular Localization of p38 in Neurons by Immunogold Electron Microscopy Cytochemistry

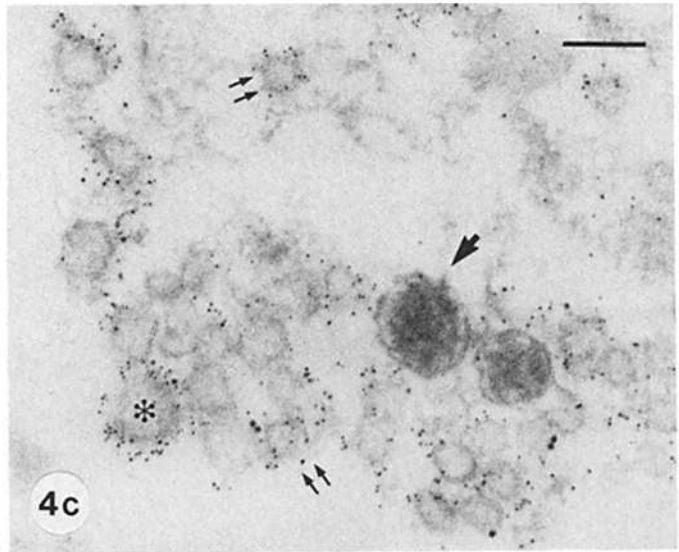
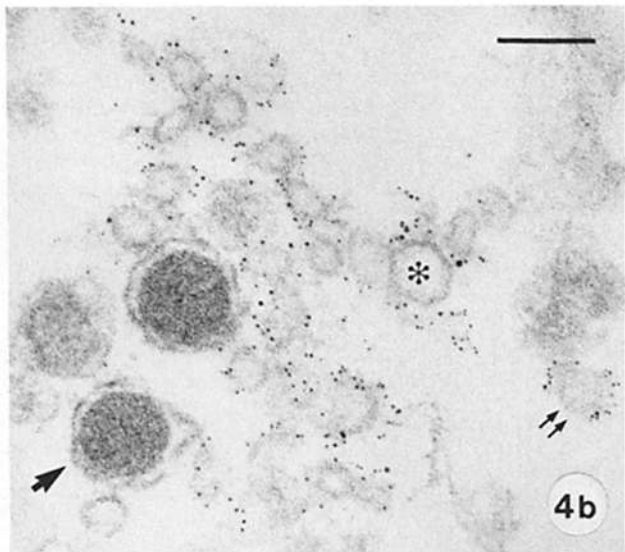
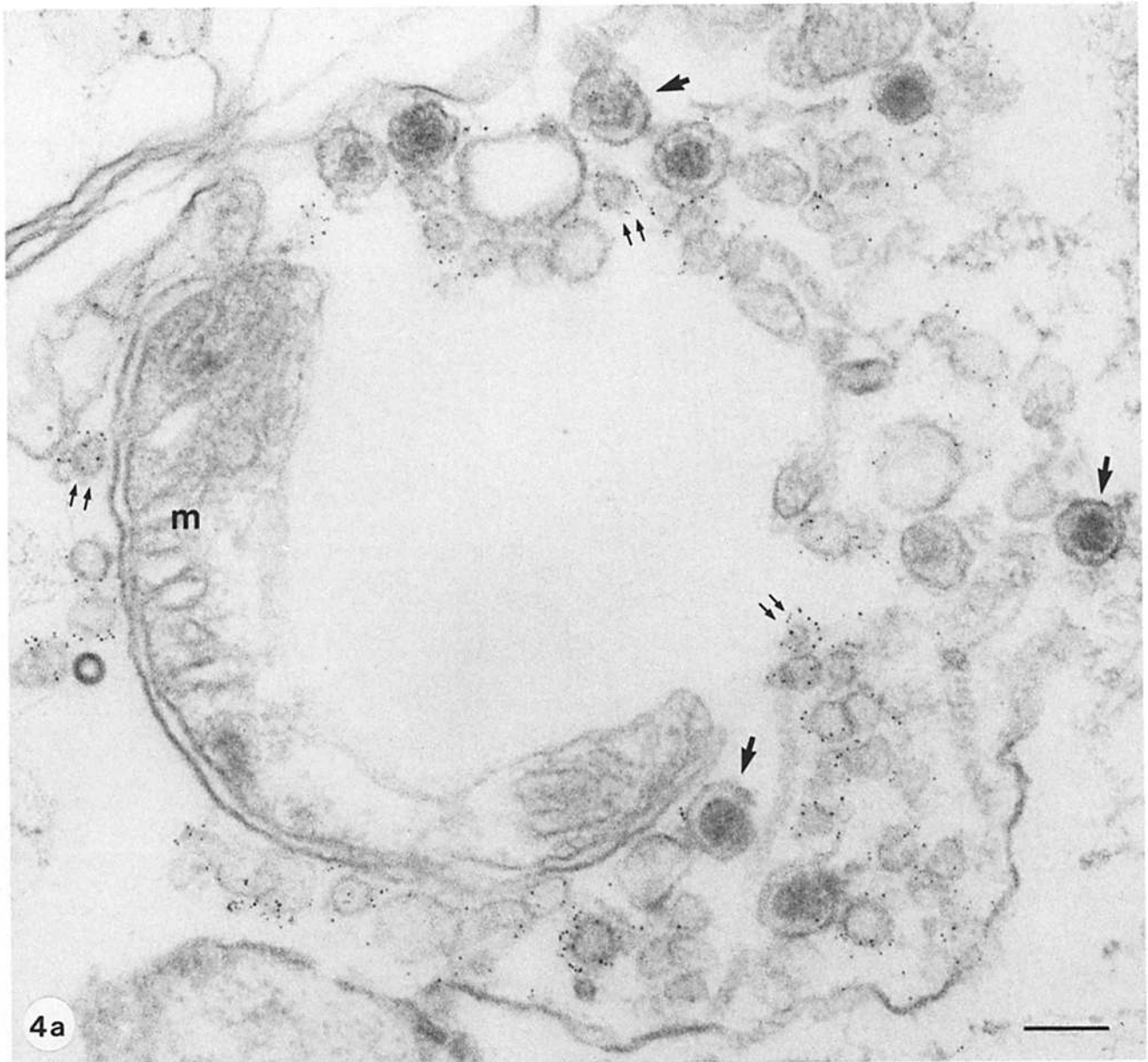
To study the distribution of p38 in brain tissue at the electron microscopic level by immunogold, we used a technique that involves labeling of subcellular particles embedded in an agarose matrix (10). As in a previous study of synapsin I (32), we used the bovine hypothalamus for these experiments. The hypothalamus was chosen because of its high content of LDCVs, the bovine species because of the relatively large size of the hypothalamus. Embedding total homogenates (32), rather than synaptosomal fractions (10), in agarose allowed us to investigate the distribution of p38 not only in nerve endings but also in other subcellular organelles. When the homogenization of the tissue was performed under gentle conditions, undisrupted cell fragments and clusters of organelles still arranged in what appeared to be the original *in situ* configuration could be observed in the final embedded preparation.

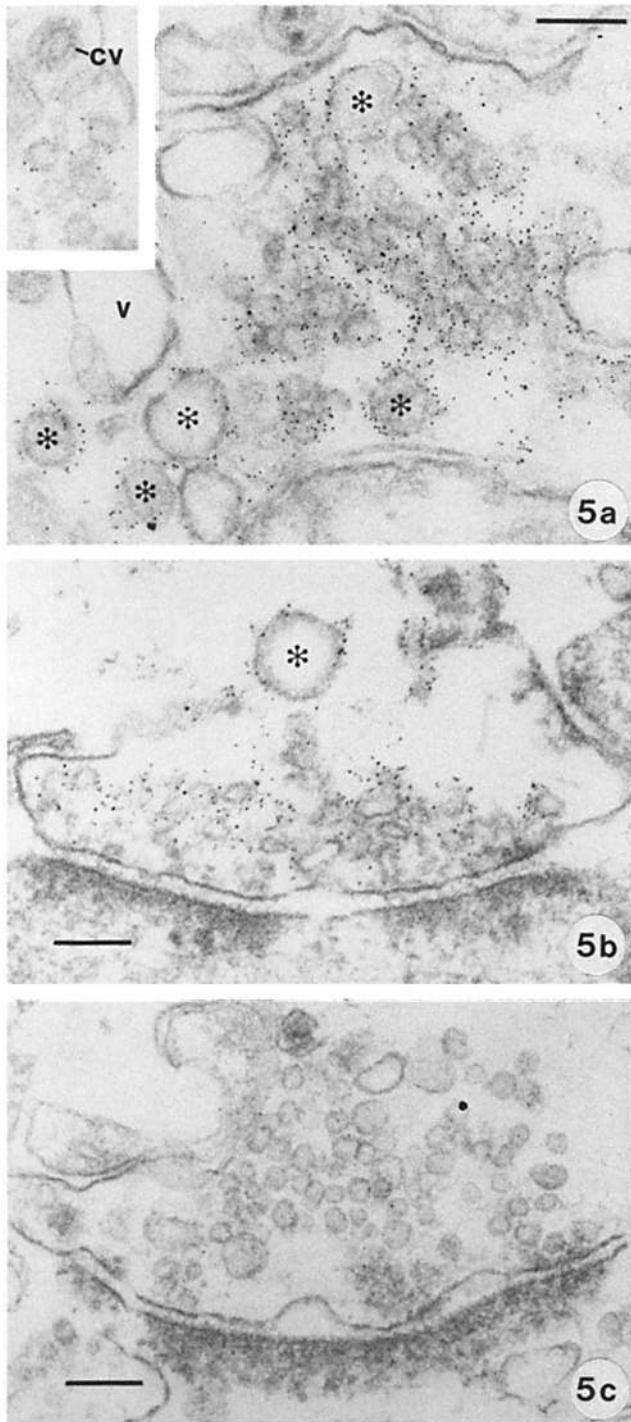
Both lytic (hypotonic) and nonlytic (isotonic) fixation con-



**Figure 3.** Relative amounts of p38 and synapsin I in various regions of the rat brain as quantitated by a dot-immunobinding assay. For comparison, the values obtained per mg of cortex protein were defined as 100. The figure shows a representative experiment.

**Figure 1.** Comparison of the localization of p38 (*a* and *c*) and synapsin I (*b* and *d*) immunoreactivities in the rat cerebellar cortex (*a* and *b*) and in the CA3-CA4 region of the rat hippocampus (*c* and *d*). 1- $\mu$ m thick plastic sections (a few micrometers apart) stained by immunofluorescence were used for comparison. In all the micrographs, fluorescent dots, which vary in size, represent individual nerve terminals. (*a* and *b*) In both fields, tightly apposed immunoreactive dots visible in the molecular layer (*ML*) represent individual nerve endings. Their bright fluorescence outlines the negative image of dendritic branches and cell somata. A thick arrow points at the unstained main dendritic trunk of the same Purkinje cell. The surfaces of Purkinje cell perikarya are discontinuously outlined by immunofluorescent dots representing nerve endings of axosomatic synapses (*arrowheads*). Large dots in the granule cell layer (*GL*) represent the large cerebellar mossy fiber terminals (*thin arrows*) which terminate in the glomeruli. *V*, blood vessel. (*c* and *d*) Large dots concentrated in the bottom part of the stratum radiatum (*SR*), just above the layer of pyramidal cell perikarya in the stratum pyramidale (*SP*), represent the large hippocampal mossy fiber terminals making synaptic contacts primarily with the proximal portion of the apical dendrites of pyramidal cells. The mossy fiber terminals seen in the stratum pyramidale in *d*, but not in *c*, are attributable to the slightly different regions of the stratum pyramidale shown in the two fields. Thin arrows point to the unstained distal segments of pyramidal cell apical dendrites which are outlined by very fine immunoreactive endings. *SO*, stratum oriens. Bar, 40  $\mu$ m.





**Figure 5.** Electron micrographs showing the localization of p38 on organelles of agarose-embedded lysed nerve endings of bovine hypothalami. Immunogold labeling of homogenates fixed under hypotonic conditions. *a* and *b* show that, in addition to labeling SSVs, immunogold also labels, to a variable extent, larger vacuoles (asterisks). Gold particles on these vesicles are located at the cytoplasmic surface. Other organelles of the ending, including the

plasmalemma, are unlabeled. The inset of *a* shows a detail of lysed nerve ending which includes a coated vesicle (cv). The latter is unlabeled. (*c*) Nerve ending from a preparation reacted with control-IgGs rather than p38-IgGs. No relevant labeling is visible. Bars: (*a*) 100 nm; (*b* and *c*) 150 nm.

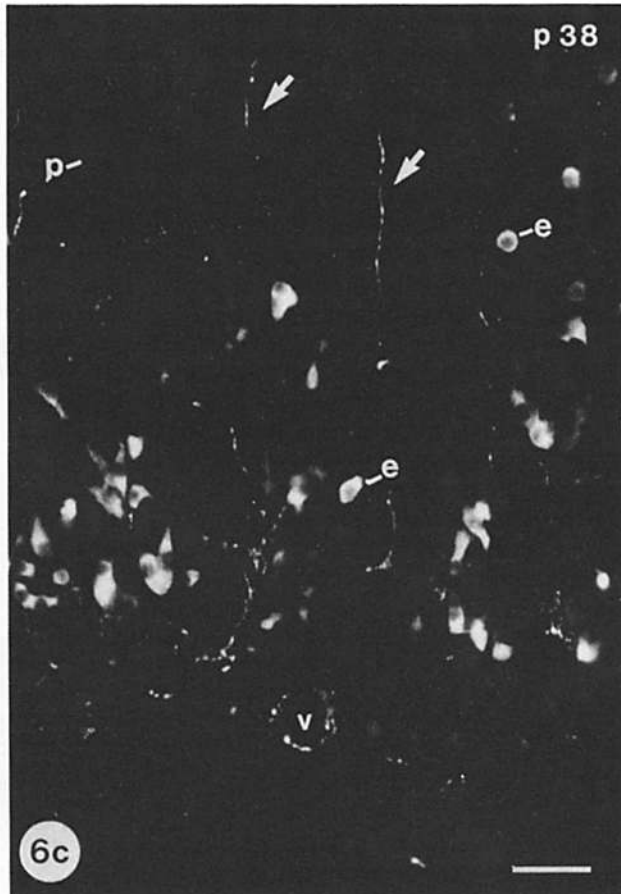
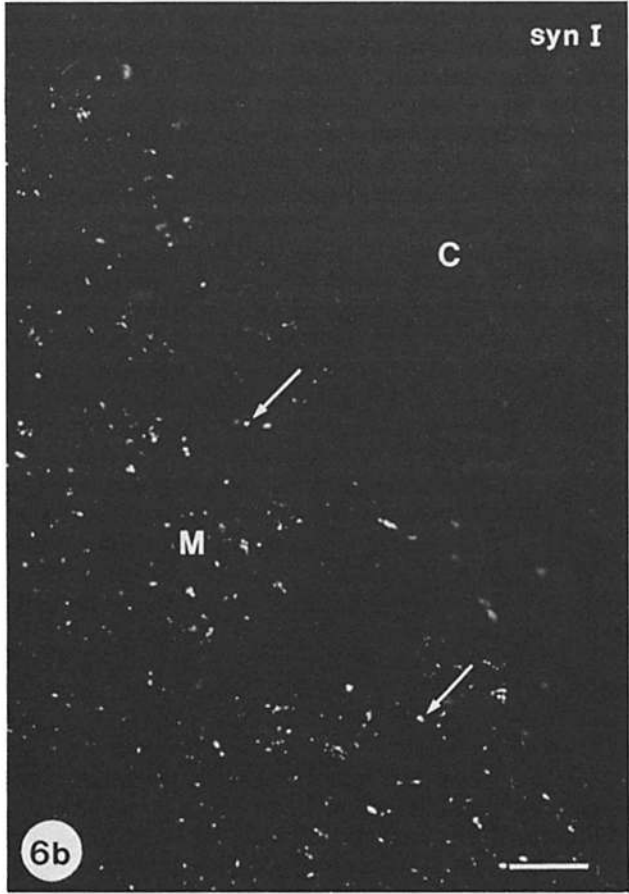
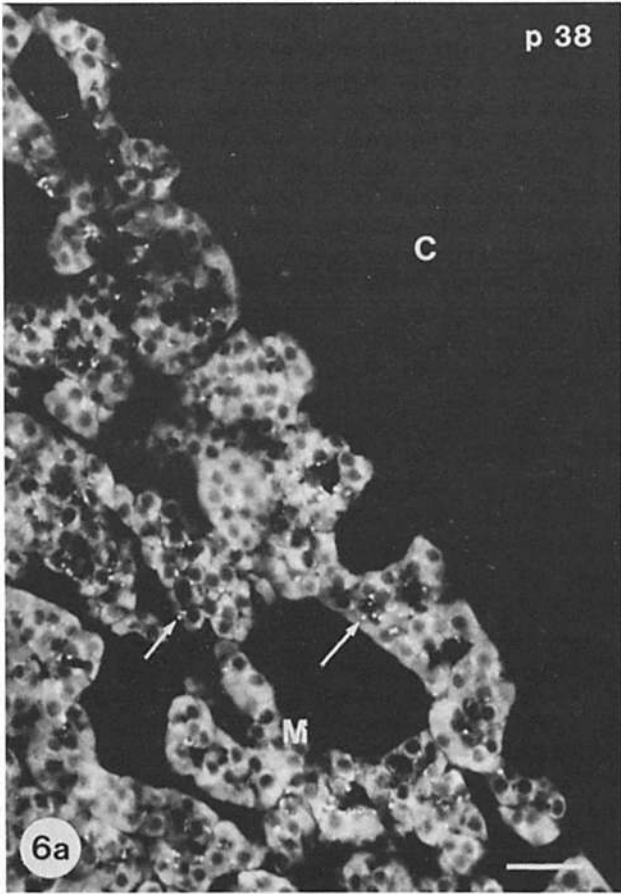
largest vacuolar profiles such as that marked by *v* in *a*, and the plasmalemma, are unlabeled. The inset of *a* shows a detail of lysed nerve ending which includes a coated vesicle (cv). The latter is unlabeled. (*c*) Nerve ending from a preparation reacted with control-IgGs rather than p38-IgGs. No relevant labeling is visible. Bars: (*a*) 100 nm; (*b* and *c*) 150 nm.

ditions were used. The lytic fixation was aimed at opening all nerve endings resealed after the homogenization, which represented more than 50% of the nerve endings in a total homogenate, and which would otherwise have been inaccessible to immunological probes (10).  
The bulk of p38 immunoreactivity was found to be localized in nerve endings (Figs. 4 and 5) and all lysed nerve endings were heavily labeled. Inside nerve endings, the large majority of gold particles was associated with the cytoplasmic surface of SSVs. Labeling of individual vesicles was variable, possibly as a result of variable accessibility (due to the presence of nearby organelles or of cytoplasmic matrix). Some vesicles were heavily labeled and as many as 30 gold particles were counted around individual SSVs. In contrast to the heavy labeling of SSVs, LDCVs were consistently unlabeled (Fig. 4). Whenever clusters of gold particles were visible in proximity to the surface of LDCVs, they could be ascribed to nearby SSVs. Lack of labeling of LDCVs was so striking that a morphometric analysis of the differential labeling of SSVs and LDCVs (as had been carried out for synapsin I [32]) did not appear to be necessary.

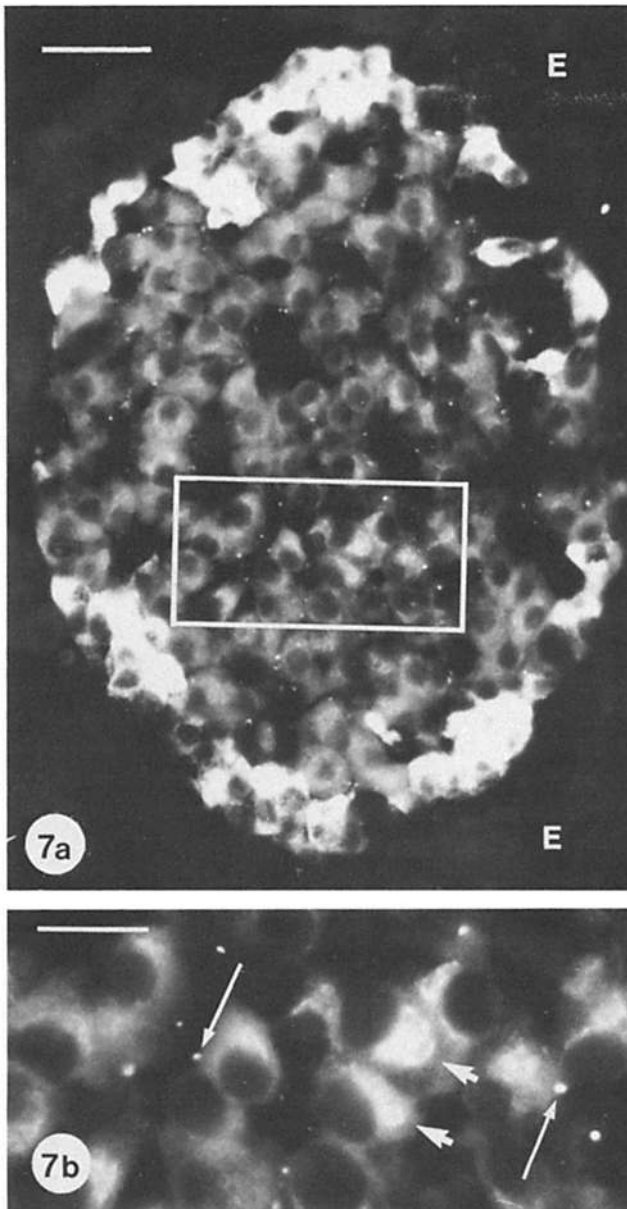
Sparse to intense immunolabeling was also observed on the cytoplasmic surface of some vesicle profiles larger than SSVs and without a dense core (Figs. 4, *b* and *c* and 5, *a* and *b*). These might represent intermediate stages in the process of re-formation of SSVs after endocytosis (19). Most large vesicular profiles of nerve endings, however, were unlabeled. Few coated vesicles were seen in our preparations (10, 20). Those that were seen were usually unlabeled (Fig. 5 *a*, inset). This is in contrast to the previously reported observation that p38 is enriched in brain coated vesicles (36, 49), but is in agreement with a previous morphological study (47). Lack of labeling on coated vesicles might reflect poor accessibility of the vesicle membrane to immunoreagents due to the presence of the coat. The plasmalemma, both synaptic and extrasynaptic, was consistently unlabeled, suggesting the existence of mechanisms that prevent intermixing of membrane components upon SSV exocytosis (cf. reference 46).

Apart from vesicles in nerve endings, only a few other smooth-surfaced vesicular profiles present in the homogenate were decorated by immunogold in the agarose-embedded preparations (not shown). Most of these particles were small vesicles. All other subcellular particles, including large plasmalemma sheaths and rough endoplasmic reticulum membranes, were not labeled above background. None of the labeling described with p38-IgGs was seen in preparations processed with control-IgGs (Fig. 5 *c*).

**Figure 4.** Gallery of electron micrographs showing details of lysed nerve endings and illustrating the selective association of p38 with SSVs. Immunogold labeling of agarose-embedded bovine hypothalamic homogenates fixed in hypotonic (*a* and *b*) or isotonic (*c*) conditions. Immunogold particles are selectively localized and concentrated on the cytoplasmic surface of SSVs (pairs of small arrows). Besides SSVs, the only labeled organelles are some vacuoles which are slightly larger than the average SSVs and have a clear content (asterisks). LDCVs (large arrows) and all other organelles are unlabeled. *m*, mitochondrion. Bars, 100 nm.

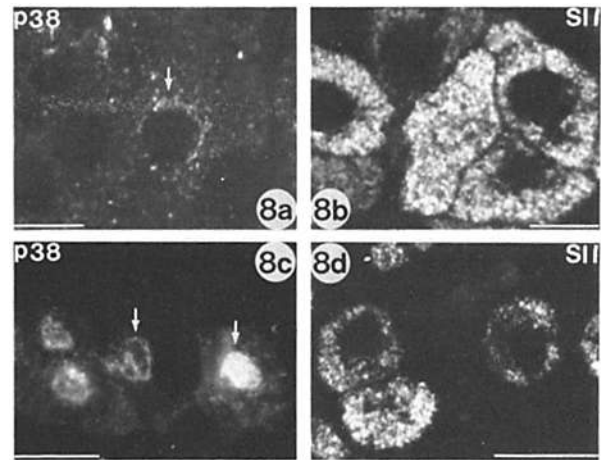






**Figure 7.** Localization of p38 by immunofluorescence in frozen sections of the rat pancreas. (a) Low power view of a pancreatic islet. P38 immunoreactivity is detectable in all endocrine cells of the islet but is more concentrated in cells localized at its periphery. This may reflect a difference in concentration of p38 in cells that produce different types of pancreatic hormones. The exocrine pancreas (E) is unstained. (b) High power view of the field enclosed by the rectangle in a. The figure shows that two patterns of immunoreactivity coexist in individual endocrine cells. One consists of a diffuse cytoplasmic stain, the other of a more intense particulate stain localized in a paranuclear region (short arrows). Long arrows point at immunoreactive nerve terminals. Bars: (a) 35  $\mu\text{m}$ ; (b) 15  $\mu\text{m}$ .

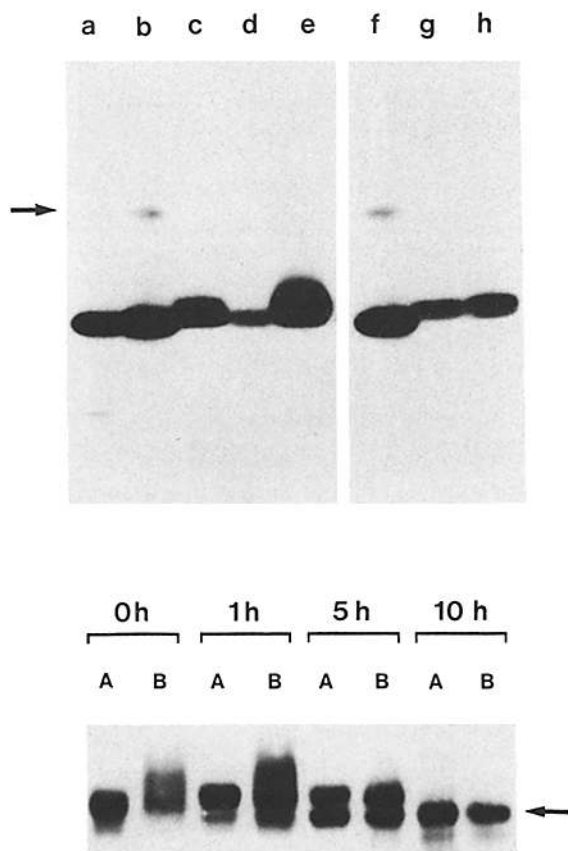
**Figure 6.** Comparison of the distribution of p38 (a and c) and synapsin I (b and d) immunoreactivity in the rat adrenal gland (a and b) and the mucosa of the rat stomach (c and d). Frozen sections stained by immunofluorescence. (a and b) Bright fluorescent puncta (arrows) scattered among chromaffin cells of the adrenal medulla are visible in sections stained either for p38 or for synapsin I. They represent axon terminals innervating chromaffin cells. No axon terminals are visible in the adrenal cortex, which is not innervated. Chromaffin cells are p38-positive but synapsin I-negative. M, medulla; C, cortex. (c and d) Varicose fluorescent fibers visible in both fields (arrows) represent "en passant" varicose terminals innervating the gastric glands. The sparse cells scattered among the glands, which are positive for p38 but negative for synapsin I, represent endocrine cells (e) of the stomach mucosa. Exocrine cells of the gastric gland are immunoreactive neither for p38 nor for synapsin I. The low-level fluorescence visible in parietal cells (p) in both fields represents nonspecific fluorescence. v, blood vessel surrounded by immunoreactive "en passant" varicose terminals. Bars, 40  $\mu\text{m}$ .



**Figure 8.** Comparison of the distribution of p38 (a and c) and secretogranin II (b and d) immunoreactivity in the rat adrenal medulla (a and b; 1- $\mu\text{m}$  thick frozen sections) and anterior pituitary (c and d; 1- $\mu\text{m}$  thick plastic sections). In a and c a faint, finely punctate, fluorescence can be seen throughout the cytoplasm. A more intense accumulation of immunoreactivity is seen in proximity of nuclei (arrows), at the site of the Golgi complex, which has a different light microscopic morphology in chromaffin cells and in pituitocytes. In b and d, which show similar regions of the same glands, an evenly distributed population of fluorescent puncta of roughly equal size and staining intensity occupies the cell cytoplasm. Such puncta represent secretory granules. SII, secretogranin II. Bars, 10  $\mu\text{m}$ .

#### Distribution of p38 in Non-neuronal Tissues

In a survey of several non-neuronal tissues by light microscopy immunocytochemistry, p38 was found in a variety of endocrine cells. Under similar conditions of immunostaining, synapsin I immunoreactivity was consistently found only in nerve terminal-containing regions (e.g., Fig. 6; unpublished observations). Non-neuronal cells highly immunoreactive for p38 included chromaffin cells of the adrenal medulla (Fig. 6 a; see also Fig. 8 a), endocrine cells of the stomach (Fig. 6 c) and of the pancreas (Fig. 7), cells of the anterior pituitary (Fig. 8 c), and type C cells of the thyroid (not shown). The identity of the sparse p38-positive cells in the stomach and thyroid as endocrine cells was established by labeling adjacent sections with antibodies directed against secretogranin II, a secretory protein previously shown to be a marker for a variety of cells of the neuroendocrine system (39, 40). The intensity of immunostaining was not the same in all p38-positive cells (see for example the more intense immunofluorescence of the peripheral cells in the islets of Langerhans; Fig. 7 a), indicating differences in the concentrations of p38 in different subpopulations of endocrine cells. With the possible exception of parotid acinar cells, in which a low level of immunoreactivity was discerni-



**Figure 9.** Presence of p38 immunoreactivity in non-neuronal tissues, and relationship between neuronal and non-neuronal p38 as revealed by deglycosylation of the molecule. (*Top*) Immunoblot of total homogenates of rat and bovine tissues and of two cell lines. The protein samples were separated on a 9–13% linear gradient SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was labeled with p38-IgGs and the immunoreactive bands visualized using  $^{125}\text{I}$ -protein A. Lane *a*, Purified rat brain p38 (12.5 ng of protein); lanes *b–h*, homogenates (10  $\mu\text{g}$  of protein per lane) of: (*b*) rat brain cortex; (*c*) rat anterior pituitary; (*d*) GH<sub>4</sub>-cells; (*e*) PC12 cells; (*f*) rat brain cortex; (*g*) bovine adrenal medulla; (*h*) bovine adrenal chromaffin cells. The band labeled by an arrow corresponds to the dimeric form of p38 (see Materials and Methods). Note that p38 immunoreactivity in non-neuronal tissues has a slightly lower electrophoretic mobility than brain p38. (*Bottom*) Deglycosylation of p38 purified from rat brain (lanes *A*) and from PC12 cells (lanes *B*), showing that the protein core of p38 has the same electrophoretic mobility. Deglycosylation was carried out by trifluoromethanesulfonic acid. The reaction was carried out for the time indicated at the top of the lanes. The samples were separated on a 9–13% linear gradient SDS polyacrylamide gel (3  $\mu\text{g}$ /lane) and transferred to a nitrocellulose membrane. The blot was labeled with p38-IgGs and the immunoreactive band was visualized using immunoperoxidase. The figure shows the p38-containing region of the gel. The arrow points to the position of the deglycosylated form of p38.

ble, no cells other than those of the neuroendocrine system were found to be immunoreactive.

The presence of p38 in a variety of tissues was also investigated by blot analysis of total homogenate proteins. Outside the brain significant amounts of p38 immunoreactivity were detected in tissues composed primarily of neuroendocrine cells, namely the adrenal medulla and the anterior pituitary

(Fig. 9, top). p38 immunoreactivity was also highly concentrated in cell lines derived from these two tissues, namely PC12 and GH<sub>4</sub> cells (Fig. 9, top). Liver, skeletal muscle, several exocrine glands (pancreas, submaxillary, and lacrimal glands), lung, kidney, and testes were either found to be negative or to contain only faint immunoreactivity (attributable either to their innervation or to the presence of a small proportion of neuroendocrine cells). A significant amount of p38-like immunoreactivity could be detected in the parotid gland with the serum antibody (not shown); however, none of the four monoclonal antibodies tested cross-reacted with this band. In all other tissues, identical results were obtained with serum and monoclonal antibodies.

As can be seen from Fig. 9 (top), the protein band cross-reacting with the p38-IgGs in endocrine tissues exhibited a slightly higher apparent molecular weight than did brain p38. To establish whether this difference was due to a difference of the protein core or of the sugar part of the molecule, p38 fractions isolated from rat brain and from PC 12 cells were deglycosylated in parallel incubations using trifluoromethanesulfonic acid. The results of one such experiment are shown in Fig. 9 (bottom). During the course of the reaction, in both brain and PC 12 samples, an identical protein band of  $\sim 34,000$  D appeared (arrow), which was apparently generated from the higher molecular weight form. The reaction seemed to be complete after 10 h of incubation since extended treatment did not further change the pattern (not shown).

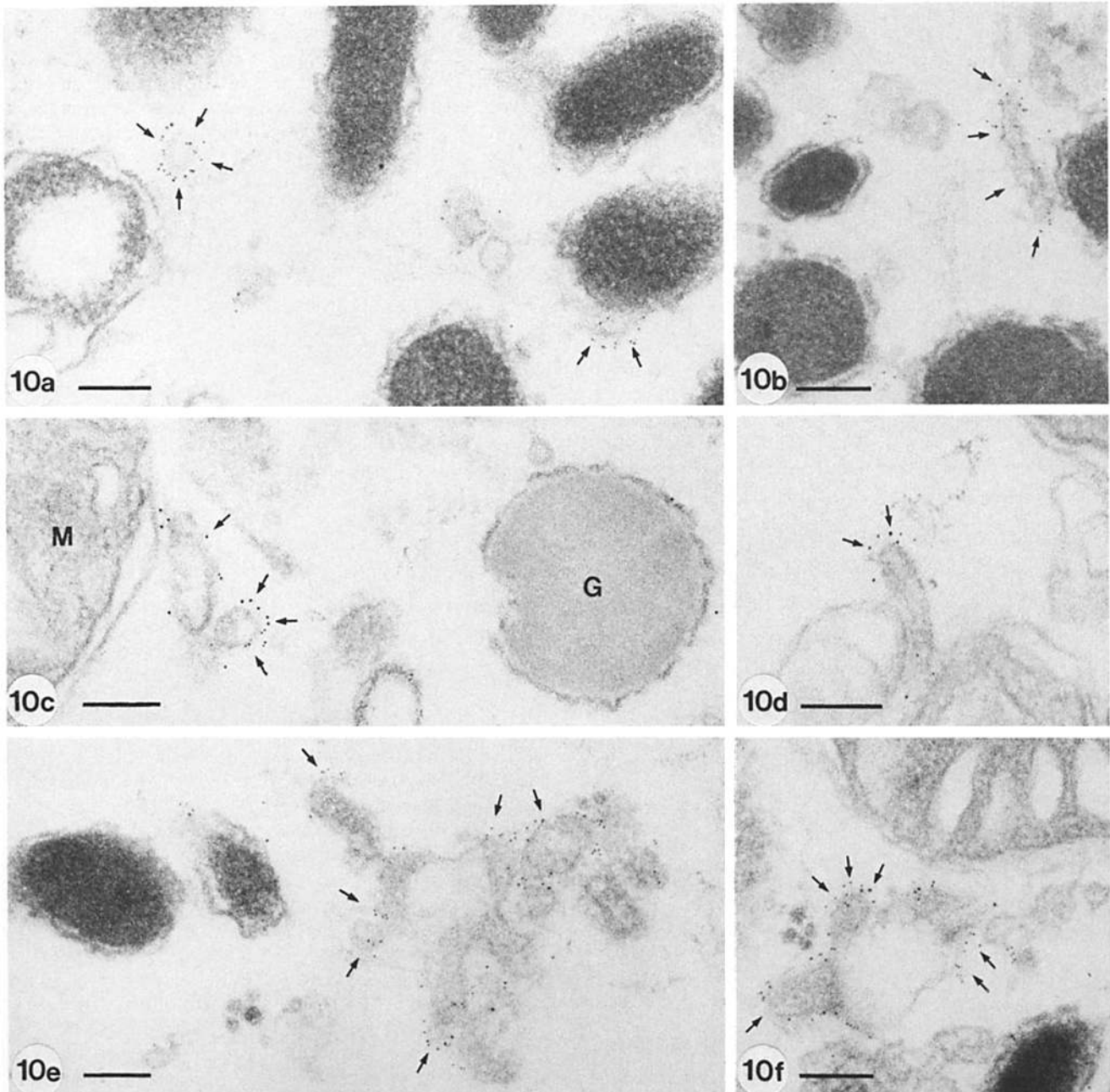
#### Subcellular Localization of p38 in Non-neuronal Cells

**Light Microscopy Immunocytochemistry.** High power observation of tissue sections stained for p38 by immunofluorescence revealed that the immunoreactivity for this protein was unevenly distributed within endocrine cells. A moderate to high level of immunoreactivity was visible throughout the cytoplasm (Figs. 7 *b* and 8, *a* and *c*). In addition, a more intense immunoreactivity was visible in the vicinity of the nucleus (Fig. 7 *b* and 8, *a* and *c*). The distribution of this paranuclear material varied in different types of endocrine cells and was reminiscent of the light microscopic appearance of the Golgi complex characteristic of that given cell type (for example, reference 34, unpublished observations).

Comparison of the staining patterns obtained after immunostaining for p38 and secretogranin II (used here as a marker for secretory granules) (39, 40) revealed a striking difference in the distribution of the two proteins (Fig. 8). Secretogranin II immunoreactivity occurred in the form of very bright dots tightly packed throughout most of the cytoplasm. The texture of this punctate staining appeared quite different from the texture of the p38 staining.

**Electron Microscopy Immunocytochemistry.** To further identify the precise subcellular localization of p38 in endocrine cells, we studied the distribution of p38 immunoreactivity in two cell types, the pituitocytes of the rat anterior pituitary and the chromaffin cells of the bovine adrenal medulla, by two different and complementary immunogold electron microscopy techniques.

(*a*) **Adrenal Medulla:** In chromaffin cells of the adrenal gland, the distribution of p38 was studied by the same procedure used to determine the subcellular localization of p38 in the brain (see above), i.e., by immunolabeling total homogenates embedded in agarose. Bovine adrenal medullae were



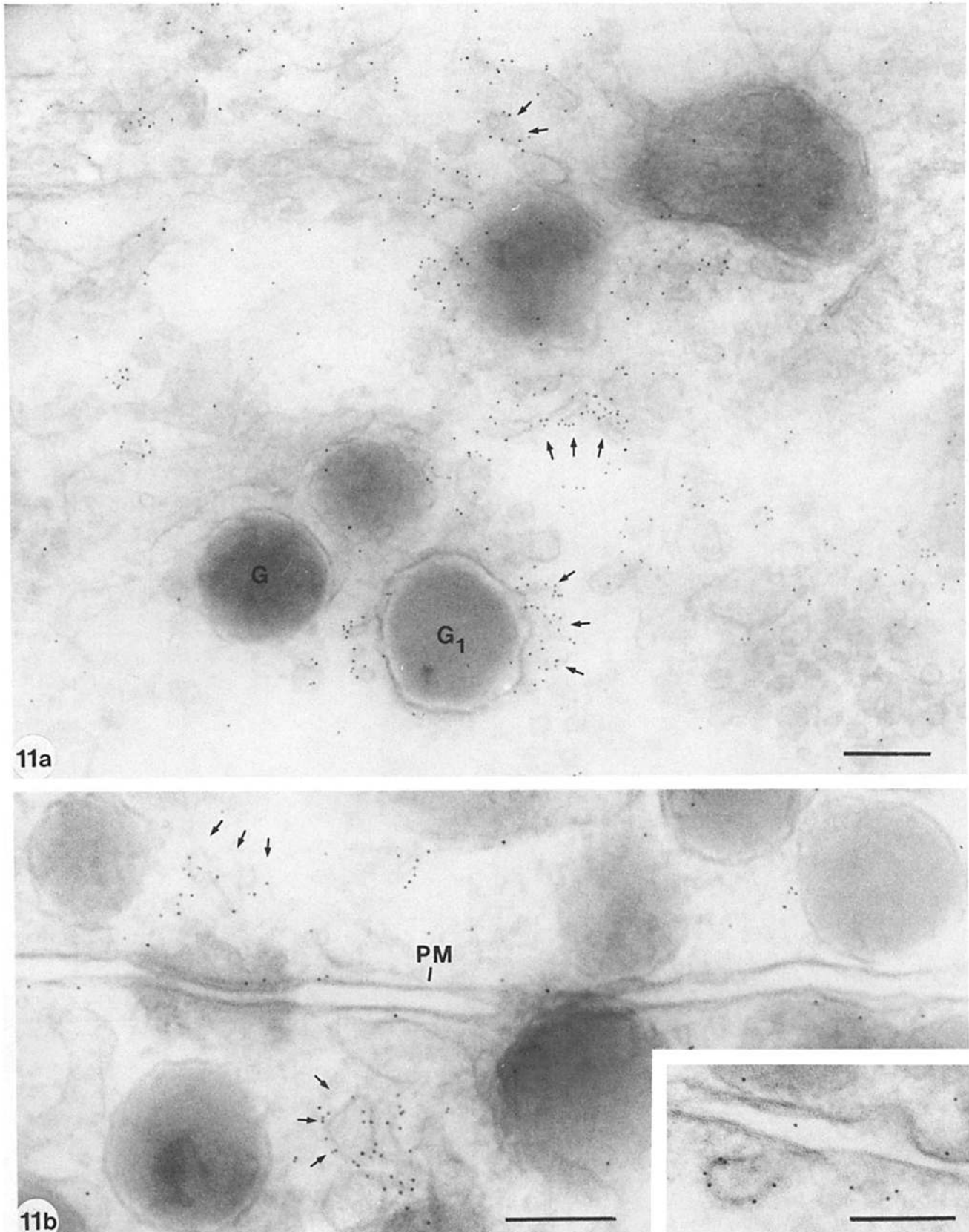
**Figure 10.** Agarose-embedded subcellular particles of bovine adrenal medulla homogenates labeled by immunogold for p38. The figure shows a gallery of various types of membrane profiles which are p38 positive (arrows). These profiles are either small round or oval vesicles (a and c), small tubules (b and d), or larger, highly infolded vacuoles (e and f). Membranes of secretory granules (G), mitochondria (M), and other organelles are unlabeled. The different staining intensity of the core of secretory granules in the various fields is the result of different times of counterstaining. Bars, 100  $\mu$ m.

used for these experiments. In these preparations, p38-immunogold selectively labeled a pleiomorphic population of smooth-surfaced membranes. These were predominantly round or oval vesicles with a dimension in the same range as that of small synaptic vesicles of neurons (Fig. 10). Occasionally, small tubular profiles (Fig. 10, b and d) and larger structures with highly infolded profiles (Fig. 10, e and f) were also seen. Most of the labeled vesicular surfaces were randomly interspersed with other organelles, but clusters of them were consistently seen in proximity to centrioles and

to remnants of the Golgi complex (piles of a few flattened cisternae) (not shown).

In general the labeling density on all p38-positive membranes was high. Only rare, scattered, gold particles were seen on secretory granules. Immunogold was virtually absent from all other organelles. None of the specific label described was observed in preparations reacted with control-IgGs instead of p38-IgGs.

**(b) Anterior Pituitary:** In the anterior pituitary the subcellular localization of p38 was studied by surface labeling of



**Figure 11.** P38 immunoreactivity in the rat anterior pituitary as revealed by immunogold labeling of ultrathin frozen sections. (a) The great majority of gold particles appear to be associated with small vesicular profiles with clear content (*arrows*). These are interspersed among secretory granules (*G*), sometimes in close proximity to them (*G*<sub>1</sub>). (b) p38-positive vesicles (*arrows*) are visible in the proximity of the plasmalemma. Only scattered particles are visible on most regions of the plasmalemma itself. The inset of *b* shows two plasmalemma invaginations, one of which is labeled by several gold particles. Bars, 150 μm.

ultrathin frozen tissue sections. These experiments complemented results obtained with the adrenal medulla because they allowed one not only to establish the identity of p38-positive organelles by another technique, but also to determine their distribution in the context of intact cells. Some results are illustrated in Figs. 11 and 12 *a*.

By far the majority of immunogold particles were associated with smooth-surfaced small vesicular profiles scattered among secretory granules and concentrated in close proximity to the Golgi complex. These vesicular profiles had variable shapes ranging from small round or oval vesicles to irregularly shaped vacuoles and short tubules. Not all smooth-surfaced vesicular profiles appeared to be labeled and, even at the para-Golgi region, clusters of labeled vesicles were interspersed with clusters of unlabeled vesicles. Very few gold particles were seen on membranes of the rough endoplasmic reticulum (Fig. 12 *a*), on membranes of the major Golgi stacks (Fig. 12 *a*, *inset*), or on profiles of secretory granule membranes (Figs. 11 and 12 *a*). Only scattered gold particles were visible on the plasmalemma (Fig. 11 *b*), but a high density of particles was occasionally seen at plasmalemma invaginations (Fig. 11 *b*, *inset*). Mitochondria were consistently unlabeled.

The specificity of the labeling was assessed by two controls. Sections adjacent to those immunolabeled for p38 were immunolabeled with either control-IgGs or with IgGs directed against secretogranin II. The labeling described above was totally absent in sections reacted with control-IgGs. The distribution of secretogranin II immunoreactivity was completely different from the distribution of p38 immunoreactivity. The labeling pattern for this antigen is illustrated in Fig. 12 *b*, which shows a cytoplasmic region including the Golgi complex. Practically all immunogold particles were concentrated on the core of secretory granules.

## Discussion

### *p38 in Neurons*

Two previous studies demonstrated the presence of a major synaptic vesicle protein p38 (also referred to as synaptophysin [47]) in terminals of several regions of the nervous system (22, 47). In this study we show both by immunohistochemistry and by a quantitative immunoassay that in nearly all brain regions p38 has a distribution very similar to that of synapsin I (9). Since synapsin I is known to be present in virtually all nerve endings (9, 10), our results suggest that p38 also has a ubiquitous distribution in nerve endings. The stoichiometric ratio of the two proteins is close to 1. A discrepancy between the nerve terminal distribution of p38 and synapsin I was noted only at highly specialized synaptic regions, such as the inner and outer plexiform layers of the retina (47; our unpublished results) and the vestibular neuroepithelium (Favre, D., D. Corey, G. Di Gioia, and P. De Camilli, unpublished observations). A separate study of such specialized regions of the nervous system is in progress to determine whether the observed discrepancy is due to a lack of synapsin I, but not of p38, from ribbon synapses (see also reference 8).

Immunogold electron microscopy indicated that, at least in the hypothalamic nerve endings examined in this study, p38, like synapsin I (32), is localized on SSVs but not on LCDVs. Work in progress in our laboratories suggests that

such a selective association of p38 with SSVs is general in the nervous system. By both immunocytochemistry and subcellular fractionation we found that, in terminals of the posterior pituitary, p38, like synapsin I (32), is selectively associated with 40–60-nm diam vesicles but not with neurosecretory granules (unpublished results). The function of 40–60-nm diam vesicles in the posterior pituitary is still unknown, but they resemble in many respects SSVs of the brain (3). Further evidence for a selective association between p38 and SSVs was provided by comparative immunofluorescence of adjacent sections of various brain regions using antibodies directed against p38, synapsin I and secretogranin II (40). Secretogranin II is co-stored in secretory organelles with peptide neurotransmitters and can therefore be used as a marker for LDCVs. p38 and synapsin I staining patterns were always coincident, but at many sites puncta positive for secretogranin II and negative for both synapsin I and p38 were observed (Navone, F., G. Di Gioia, A. Zanini, and P. De Camilli, unpublished observations). These puncta probably represent individual LDCVs.

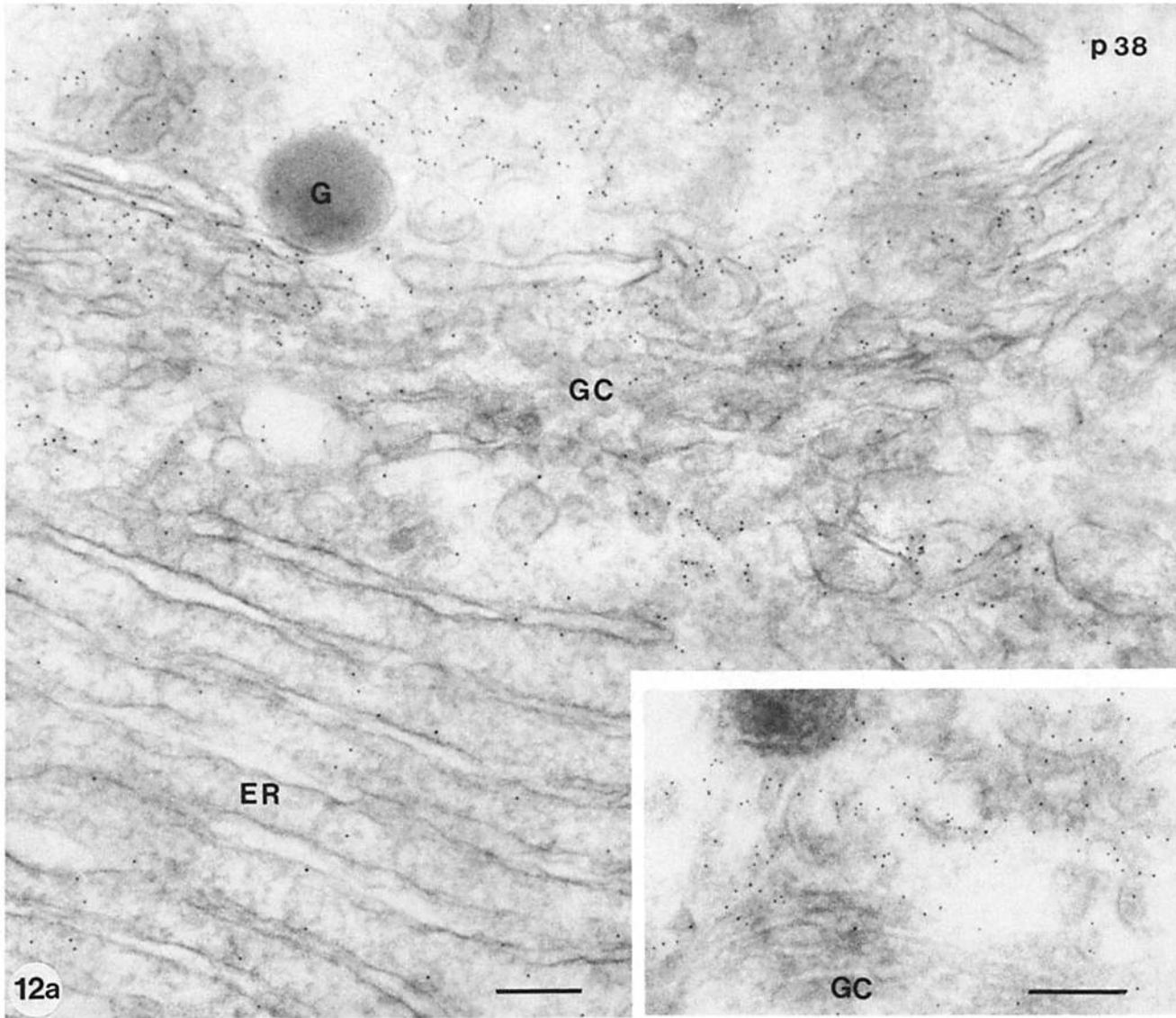
Our results indicate that the membranes of the two subpopulations of secretory organelles of nerve endings, SSVs and LDCVs, have distinct compositions and that at least one extrinsic membrane protein (synapsin I) and one intrinsic membrane protein (p38) are shared by virtually all SSVs irrespective of their neurotransmitter content.

The moderate perinuclear staining produced by p38-IgGs suggests that a minor pool of neuronal p38 is concentrated in the area of the Golgi complex. p38 in the Golgi complex might represent either newly synthesized p38 or p38 recycled from nerve terminals (17). In contrast to this perikaryal distribution of p38, the low level of synapsin I immunoreactivity visible in perikarya has a diffuse distribution.<sup>2</sup> This observation suggests that binding of synapsin I (which is known to be synthesized on free ribosomes [13]) to p38-containing membranes takes place distally to the Golgi complex. So far we have no precise indication as to the site at which synapsin I becomes associated with p38-containing membranes.

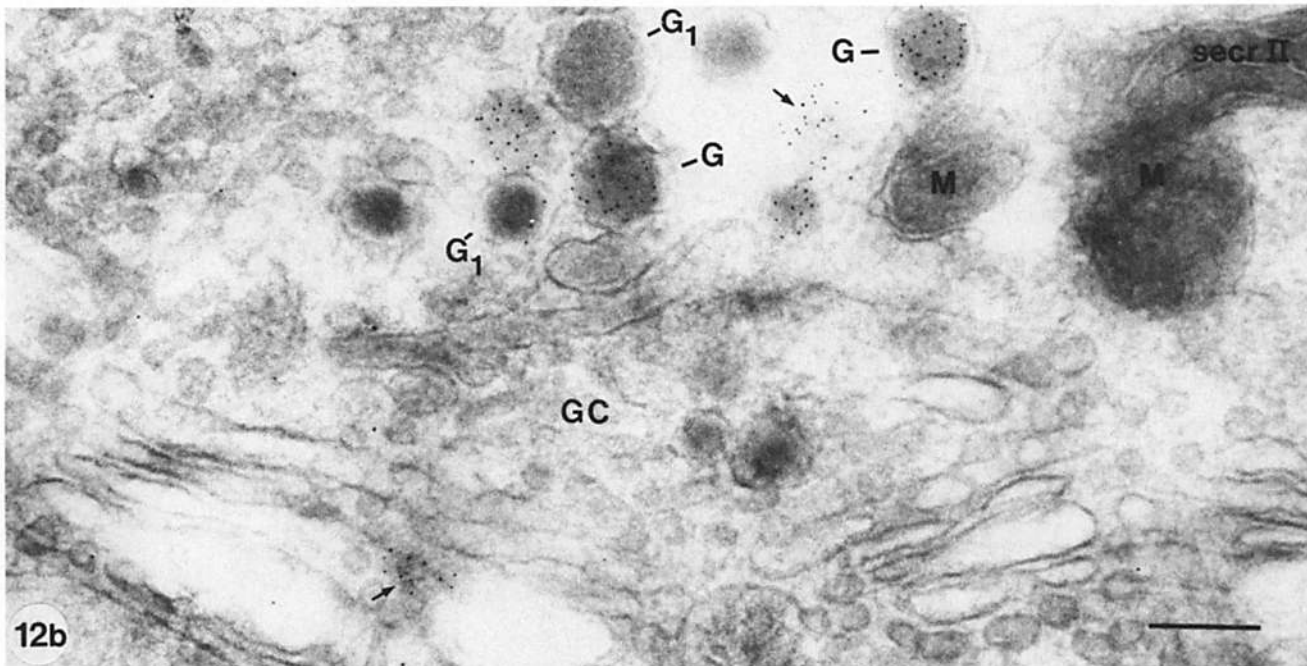
### *p38 in Endocrine Cells*

In contrast to synapsin I, which is present only in neurons, p38 is also present in a variety of endocrine cells specialized for regulated secretion of peptides via secretory granules, i.e., via the "established" pathway for regulated secretion (23, 31). This distribution of p38 suggests that it might be a com-

2. Goldenring et al. (16) have recently claimed that the localization of synapsin I primarily in nerve terminals is artifactual and that, if detergents are omitted from staining solutions, synapsin I immunostaining becomes very prominent in perikarya and dendrites. However, evidence indicating a predominant localization of synapsin I in nerve terminals is compelling (present study and references 8, 10, 29, and 32). This evidence is based on a variety of light microscopy techniques, including surface staining of ultrathin frozen sections, a procedure which does not involve use of detergents (12). The results of Goldenring et al., obtained by immunoperoxidase staining of nonpermeabilized thick vibratome sections, can be explained by the fact that antibodies penetrate much more readily into large structures, such as perikarya and major dendrites, which are split open by the knife cut, than they do into small cellular compartments, such as nerve endings, most of which are separated from the surface of section by a number of intervening membranes. Under the experimental conditions used by Goldenring et al., the ratio of perikaryal and dendritic staining to nerve ending staining can appear much higher because saturating levels of peroxide reaction product are reached in perikarya and dendrites before substantial staining is achieved in nerve endings.



12a



12b

ponent of most or all of a family of cells which, in addition to being specialized for the secretion of regulatory peptides, share a variety of other properties including a common origin from the neural crest (35). Endocrine cells that secrete peptide hormones via a regulated pathway and were found to be p38-negative include cardiac atrial cells (which secrete natriuretic peptides [7]) and some endocrine cells interspersed among epithelial cells in the surface epithelium of the duodenal mucosa. Cardiac atrial cells, however, do not originate from the neural crest, and it is controversial as to whether endocrine epithelial cells of the duodenal mucosa are derived from the neural crest (35). It should also be mentioned that the origin of the endocrine cells of the pancreas (which are p38-positive) from the neural crest has been questioned.

Our results differ somewhat from those reported for synaptophysin by Wiedenmann et al. (47) and Rehm et al. (37). Those authors found a more restricted distribution of synaptophysin, since they could not detect it in endocrine cells other than chromaffin cells of the adrenal.<sup>3</sup> Moreover, they found synaptophysin to be restricted in its phylogenetic distribution to mammals, whereas we have found p38 immunoreactivity to be present in all five vertebrate classes and several invertebrate species (unpublished results). These discrepancies might be due to a more limited epitope specificity of the antibodies used by those authors, and suggest that heterogeneous forms of p38 might exist in different tissues and species. In fact, the brain and endocrine forms of p38 differ in their sugar portions, because the observed heterogeneity of their electrophoretic mobilities disappeared after chemical deglycosylation. The shift in apparent molecular weight (from 38,000 to ~34,000 D) after deglycosylation is consistent with the report of Rehm et al. (37), who found a comparable reduction of the molecular weight of synaptophysin in PC12 cells after incubation with tunicamycin.

p38 in endocrine cells was not localized on secretory granules. At the light microscopic level p38 immunoreactivity did not colocalize with secretory granules as demonstrated by the different fluorescence patterns obtained after staining for p38 or for secretogranin II. This was further confirmed by results of electron microscopy immunocytochemistry which, in addition, allowed the determination of the precise intracellular localization of p38. p38 in endocrine cells was found to be selectively localized on a population of smooth-surfaced small vesicles and vacuoles with clear content, scattered among the granules, and particularly concentrated in the area of the Golgi complex. These results agree with the finding of Wiedenmann and Franke that adrenal medulla syn-

aptophysin does not co-purify with secretory granules upon subcellular fractionation (47). Those authors suggested that adrenal synaptophysin might be localized on a distinct, yet unidentified, type of organelle. In the present study, we have characterized the p38-positive organelles and shown that they are not specific to chromaffin cells of the adrenal medulla.

#### **Possible Relationships between Secretory Organelles of Nerve Endings and Organelles of Endocrine Cells**

The selective localization of p38 on SSVs in nerve endings further supports the idea that SSVs and LDCVs are secretory organelles of two distinct, and differentially regulated, secretory pathways. Furthermore, the presence of p38 on small vesicles in a variety of endocrine cells suggests that SSVs, which are regarded as neuron-specific organelles, are somehow related to an as yet uncharacterized endomembrane system present in a variety of endocrine cells, but distinct from the typical secretory granules. The finding that p38 is not present either on neuronal LDCVs or on endocrine secretory granules is in line with the concept that these two organelles are structurally and functionally related (32).

The physiological role of p38-positive vesicles in endocrine cells remains to be defined. Their accumulation in the area of the Golgi complex and the presence of p38-positive plasmalemmal invaginations suggest that p38-positive vesicles may be part of a system that functionally connects the area of the Golgi complex with the cell surface. The frequent association observed between p38-positive vesicles and centrioles further supports this interpretation, since centrioles appear to coordinate, via the microtubular network, the traffic in and out of the Golgi complex area (45). It should be noted that SSVs also provide a functional continuity between the Golgi complex area and the cell surface in nerve cells. On the other hand, p38-positive vesicles of endocrine cells clearly differ from neuronal SSVs. They are heterogeneous in size and shape, and synapsin I is not present on their surface. Furthermore, in neurons by far the largest pool of p38-positive vesicles is concentrated in close proximity to exocytotic sites, while only a small pool of p38 is present in the Golgi complex area. In contrast, in endocrine cells p38-positive vesicles are highly concentrated in proximity to the Golgi complex and do not appear to accumulate and cluster under the plasmalemma.

It is possible that neuronal SSVs and p38-positive vesicles of endocrine cells are cell type-specific variants of the same organelle. Synapsin I, a protein thought to act as a link between SSVs and the cytoskeleton (9, 32), might be involved in the adaptation of such organelles for neuronal function. It will be important to determine how many proteins of SSVs, in addition to p38, are shared by the endocrine vesicles. Two other intrinsic membrane proteins of SSVs have previously

3. After the completion of this manuscript, a study appeared reporting the occurrence of synaptophysin in the islets of Langerhans and in a variety of human neoplasms originating from neuroendocrine cells but not in any other type of normal neuroendocrine cells (48).

**Figure 12.** Ultra-thin frozen sections of rat anterior pituitary showing the Golgi area labeled by immunogold for p38 (a) and secretogranin II (b). (a) A high density of gold particles is visible in the area of the Golgi complex (GC). The thickness of the section does not allow precise identification of the individual vesicles with which the label is associated, but clearly gold particles are concentrated on regions rich in small vesicular profiles with clear content. The inset shows that major Golgi stacks are unlabeled. Only a low labeling density is visible on the rough endoplasmic reticulum (ER). G, secretory granule. (b) Gold particles are localized on the core of secretory granules (arrows) can be explained by a poor preservation of the granule core in these ultrathin frozen sections. Some granules are unlabeled (G<sub>i</sub>), consistent with a previous report of a heterogeneous distribution of secretogranin II in granules of single individual cells in the anterior pituitary (39). M, mitochondria; GC, Golgi complex. Bar, 150 nm.

been reported to be present in at least some types of endocrine cells (5, 30). It was inferred by the authors of such studies that these proteins were probably common to all secretory vesicles of neurons and to secretory granules of endocrine cells. In light of the present results, the possibility should be considered that in neurons as well as in endocrine cells these proteins might be selectively localized on the same vesicle subpopulations which are p38-positive and absent from LDCVs and endocrine secretory granules.

Rehm et al. have recently reported that p38 is a major  $Ca^{2+}$ -binding protein ( $Ca^{2+}$  being bound at the cytoplasmic domain of the molecule) and have suggested that, because of this property, p38 might play an important physiological role in regulated exocytosis from neurons (37). This is an interesting possibility, but the lack of p38 on peptide-containing secretory granules of neurons and endocrine cells argues against a general role of p38 in all types of regulated exocytosis. Furthermore it remains to be demonstrated whether p38-positive vesicles in endocrine cells undergo a regulated interaction with the plasmalemma.

Whatever the physiological function of p38-positive vesicles in endocrine cells might be, it may be assumed that some relation exists between such function and their function in neurons. Thus the study of the function of such endocrine vesicles may lead to the discovery of previously unknown functions of SSVs (in addition to that of being involved in neurotransmitter secretion) and/or of previously unknown secretory properties of endocrine cells.

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