Exocytosis from Large Dense Cored Vesicles as a Mechanism for Neuropeptide Release in the Peripheral and Central Nervous System

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EXOCYTOSIS FROM LARGE DENSE CORED VESICLES AS A MECHANISM FOR NEUROPEPTIDE RELEASE
IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEM

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Abstract

Nerve terminals often contain morphological- ly distinct populations of large (75-110 nm) and small (45-55 nm) vesicles. The small vesicles are speculated to account for release of trans- mitter quanta as they accumulate at presynaptic membranes. Large vesicles can co-store neuropep- tides and classical transmitters but their func- tion in neurotransmission has been disputed be- cause they do not appear to accumulate at chemi- cal synapses. However, there is now evidence that the large vesicles play a role in neuro- transmission or its modulation even though they may not be eminently involved in synaptic re- lease. Thus, exocytosis occurs along the synapse- lacking membranes of peripheral noradrenergic varicosities. Large vesicles may continue to function in peptide release even after the clas- sical transmitter has been depleted as demonstra- ted in the pig vas deferens. Three days of reser- pine administration causes a parallel loss of noradrenaline and small vesicle contents but does not decrease enkephalin-like immunoreactivi- ty or large vesicle electron density. In the central nervous system of the rat, where substan- ce P and enkephalin have been localized to large vesicles, exocytosis occurs from several types of terminals. The large vesicles appear preferen- tially to release their contents at morphologi- cally non-specialized sites even when characte- ristic synapses are present. Thus different mechanisms of transmitter and neuropeptide re- lease may coexist. The nonsynaptic discharge may allow substances to diffuse over a wider distance whereas release into a synaptic cleft could restrict receptor interaction.

KEY WORDS: Dense cored vesicles, exocytosis, neuropeptide release, nonsynaptic neuro-trans- mission

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Introduction

During the last decade numerous peptides have been added to the list of substances with proven or putative roles in chemical neurotrans- mission and its modulation. In contrast to the classical transmitters such as acetylcholine and noradrenaline, which appear to be stored and released from small synaptic vesicles measu- ring 45-55 nm in diameter, the neuropeptides are stored in morphologically distinct popula- tions of large dense cored vesicles which range in shape, matrix density and measure from 75 to 120 nm in diameter depending on the type of neuron involved. The hypothesis that the large dense cored vesicles are the main storage site of neuropeptides is based on analyses of subcel- lular fractions from peripheral noradrenergic and cholinergic neurons (Fried et al. 1984, 1985; Klein et al. 1982, 1984; Lundberg et al. 1981, 1984). These have shown that the large vesicles can store both peptides and classical transmit- ters but that small vesicles contain only trans- mitter. The facts that the small vesicles can store relatively high concentrations of typical transmitters such as acetylcholine (clear vesi- cles) or noradrenaline (dense cored vesicles) and that electron micrographs depicting chemical synapses only show small vesicles attached to the presynaptic membrane, whether at the neuro- muscular junction (Heuser et al. 1979) or in the central nervous system (Streit et al. 1972; Trubatch and Van Harreveld, 1981), have led to the belief that large vesicles are not eminently involved in chemical transmission.

The present studies were undertaken to find evidence for a role of large dense cored vesicles in nonsynaptic release of peptides in the peri- pheral and central nervous systems.

Materials and Methods

Preparation for electron microscopy

Tissues from larger mammals (ox, pig, man) were rapidly excised and fixed en bloc in 2.5

percent glutaraldehyde (pH 7.2), often followed by a chromium-dichromate step before osmification in order to better maintain the noradrenaline containing cores in the small vesicles. Some blocks were fixed in 4% potassium permanganate, which also reacts with small vesicle noradrenaline. Smaller mammals (rat, guinea pig) were perfused with a mixture of 2% formaldehyde and 2% glutaraldehyde, the tissues excised and post fixed as above. Some vasa deferentia from guinea pig were fixed en bloc in potassium permanganate.

The tissues were washed, some blocs stained in uranyl acetate, dehydrated and embedded in Spurr's low viscosity medium. Sections were taken with a diamond knife at a setting of 75 nm on an LKB Ultrotome, and placed on 200 mesh grids. After staining in lead citrate the sections were viewed under a Zeiss E M 10.

Preparation for immunochemistry

Blocks from pig vas deferens were fixed in 4% formaldehyde at pH 7.2 and rats were perfused by two steps according to a schedule modified from Berod et al. (1981) using 30 ml phosphate buffered saline (PBS) followed by 200 ml of cold 4% phosphate buffered (pH 6.5) and 200 ml borate buffered (pH 10-11) formaldehyde. The medulla oblongata was postfixated for one hour and infiltrated with buffered sucrose (5-10%) before freezing and sectioning at 16-20 μm in a cryostat. Sections from the different tissues were incubated in rabbit antisera to enkephaline or substance P, washed and treated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit serum.

Peripheral noradrenergic varicosities

Co-existence of opioid peptides and noradrenaline in large dense cored vesicles

Large dense cored vesicles can be isolated and purified from the bovine splenic nerve which is composed of 98 percent sympathetic C-fibers (Lagercrantz and Klein 1982). Using radioceptor (RRA) and radioimmuno-assay (RIA) methods to measure opioid peptides and high pressure liquid chromatography (HPLC) with electrochemical detection to measure noradrenaline, it has been calculated that a large dense cored vesicle (75 nm) in the terminal has the potential to release 58-100 molecules of enkephalins and 9000-16000 molecules noradrenaline (Klein and Thureson-Klein 1984; Thureson-Klein 1983, 1984). It is reasonable to hypothesize that opioid peptides associated with noradrenergic varicosities in other tissues, as observed by immunofluorescence, are also present in large dense cored vesicles.

Exocytosis from large dense cored vesicles in actively secreting nerve terminals

Human omental veins have a rich network of varicose noradrenergic nerve fibers containing a high proportion of large dense cored vesicles (Thureson-Klein et al. 1976; Thureson-Klein and Stjärne, 1981). While exocytosis from large vesicles is seldom observed in unstimulated terminals, electrical stimulation causes an increase in exocytotic events. This is particularly evident in tissues stimulated in the presence of

Fig 1: Micrographs from pig vas deferens.

1a. Noradrenergic (NA) terminal containing many peripherally located large dense cored vesicles. The electron density of the matrix is due to the presence of many substances, including noradrenaline, dopamine β-hydroxylase, (the enzyme that converts dopamine to noradrenaline and is co-released with the transmitter), chromogranins, ATP and peptides. The various sizes of the cores in the small vesicles mainly reflect different concentrations of noradrenaline precipitated by the chromate-dichromate containing fixative. An adjacent terminal (ACH) with translucent small- and a few large vesicles of low electron density (small arrows) is probably cholinergic.

1b. Reserpine administration for three days abolishes virtually all cores in the small vesicles but has little or no effect on the large dense cored vesicles.

1c. Leu-enkephalin-like immunofluorescence of varicose fibers in the circular muscle layer in a control pig.

1d. After reserpine treatment for three days leu-enkephalin-like immunofluorescence remains in the noradrenaline depleted varicosities.

1e. Met-enkephalin-like immunoreactivity is similarly maintained.

1f. A noradrenergic nerve terminal in which one large vesicle (arrow) is in a close position allowing it to fuse with the membrane in response to stimulation. A second large and a small (encircled) vesicle appear to have fused with the membrane and thus be in the initial stage of exocytosis.

1g. A large vesicle (arrow) in a terminal after three days of reserpine, appears to have fused with the membrane and releases its contents by exocytosis. ⟶

an α-receptor blocking agent (phentolamine), known to increase noradrenaline release. While under these conditions there is also an increase in exocytosis involving small vesicles which contributes to the overflow of transmitter, the large vesicles would be the most likely source of other co-released substances.

Vas deferens as a model to study co-existence of noradrenaline and peptide

Vasa deferentia in various species have been demonstrated to have an extensive supply of noradrenergic fibers (Sjöstrand 1965; Norberg et al. 1966) with distinct populations of small and large vesicles. Both large and small vesicles can store noradrenaline (Fried et al. 1981, 1985) but there is strong evidence that only the large vesicles contain neuropeptide Y as based on analyses of isolated light (small vesicles) and heavy (large vesicles) particle fractions from the rodent vas deferens (Fried et al. 1985). While the rat vas deferens has a low content of enkephalin, other species including bovine (Klein et al. 1984) and swine have high contents. Thus the pig, with 720 pmol met-enkephalin and 210 pmol leu-enkephalin/g tissue, should be a
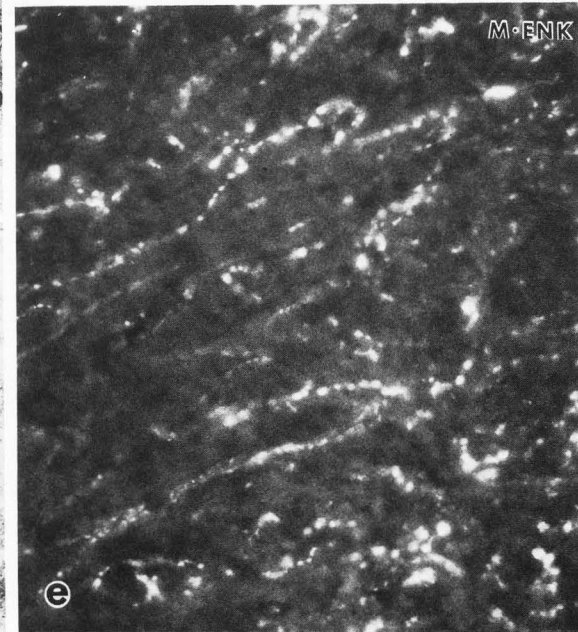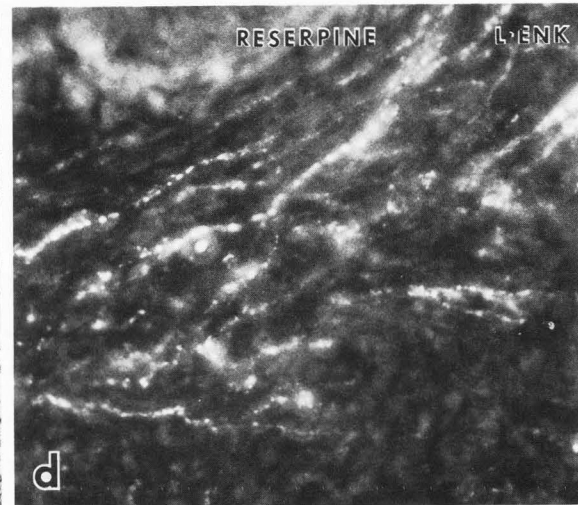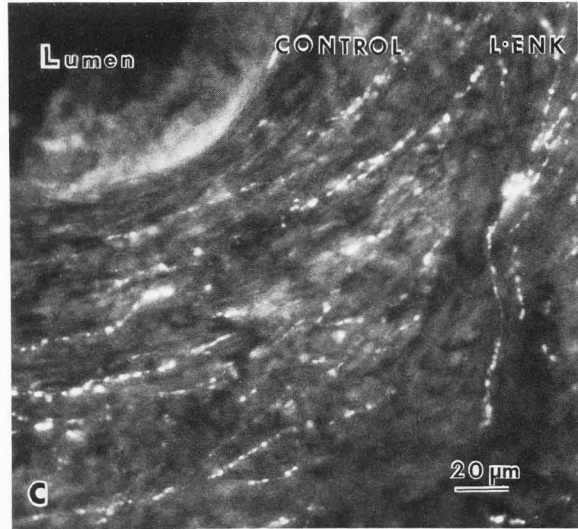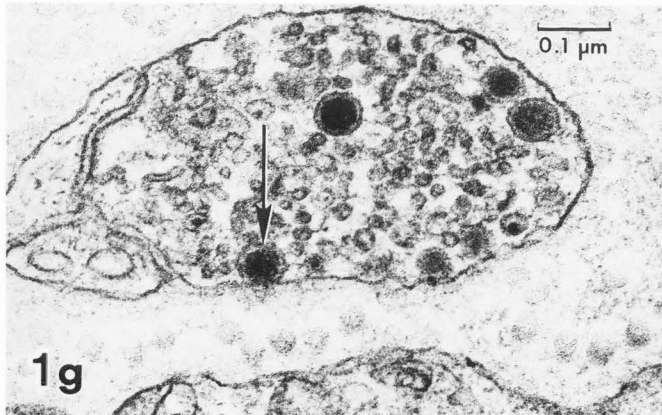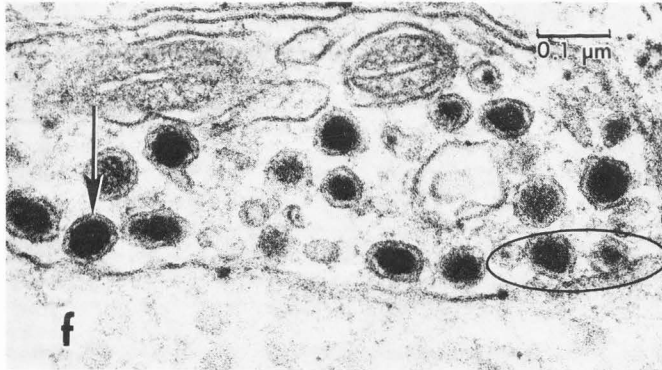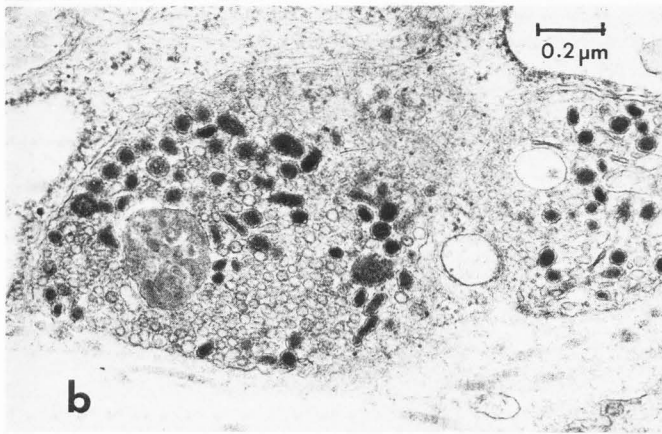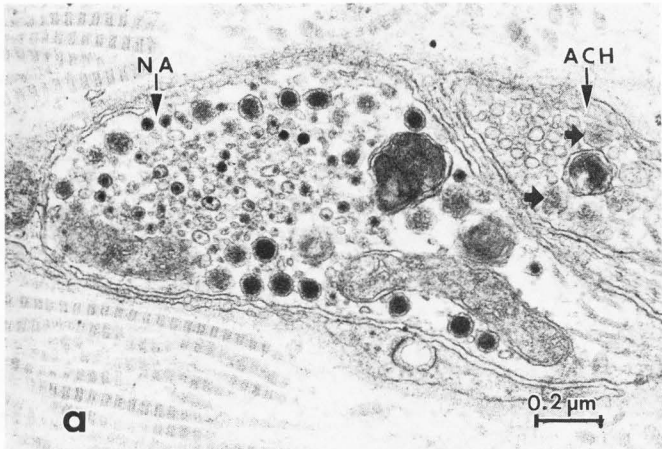
good model to study whether there is a good correlation between high numbers of large vesicles and high concentrations of opioid peptides and whether the large vesicles function in exocytotic release.

Ultrastructural examination of vasa deferentia from young pigs revealed numerous varicosities among the smooth muscle cells. The majority of these were noradrenergic based on the electron-dense staining of the core in the small vesicles which is believed to reflect noradrenaline after permanganate (Hökfelt 1969; Hökfelt and Jonsson 1968) or chromate-dichromate (Tranzer and Richards 1976) fixation (Fig 1a). A few terminals that had non-reactive, clear vesicles, were believed to be cholinergic. These often occurred adjacent to the typical noradrenergic terminals (Fig 1a).

Reserpine administration to the pig for three days resulted in almost complete loss of electron-dense cores from the small vesicles (Fig 1b). In contrast, the large vesicles maintained their electron density. Parallel immunofluorescent observations showed that the enkephalin-like immunoreactivity was similar in controls and reserpine treated animals (compare Fig 1c with d and e). There were also relatively small, if any, changes in the enkephalin content of the vasa deferentia as measured with RIA (unpublished observations).

Large dense cored vesicles sometimes had a peripheral distribution within the terminals whether in control (Fig 1a) or reserpinized (Fig 1b) animals. This may be of physiological importance because the exocytosis process requires that a vesicle is close (about 20 nm) to the terminal membrane in order to fuse in response to stimulation. Examples of large and small vesicles that appeared to be in the initial stage of exocytosis were encountered in controls (Fig 1f) as expected, but some large vesicles fused with the terminal membrane were also found in reserpine treated pigs (Fig 1g). This suggests that the large vesicles continue to function in the release of peptides even after noradrenaline depletion.

Varicosities in the central nervous system

The trigeminal nucleus caudalis as a model system

The neuropil within laminae I and II in the trigeminal nucleus caudalis portion of the medulla oblongata contains central terminals from bipolar afferent primary sensory fibers that originate from the infraorbital nerve to supply the whiskers (vibrissae) and skin on each side of the nose in the rat (Jaquin et al. 1983; Zhu et al. 1981). The whiskers are important sensory organs (Hayaski 1980) in rodents and the middle row may be supplied with as many as 130-260 sensory fibers (Waite and Cregg 1982) of which central terminals are in contact with second order neurons that project to the brain and also with intrinsic neurons, some of which may be inhibitory (Bennett et al. 1982). Thus several different categories of terminals are present and can be expected to be affected by injury or stimulation of mechano- and nociceptors

Fig 2. Micrographs from terminals in the neurophil of laminae I and II in the trigeminal nucleus caudalis demonstrating morphologically different types of large dense cored vesicles that were found to undergo preferential non-synaptic exocytosis.

2a. Portion of a long slender terminal or varicose fiber (arrows) containing ovoid to round large vesicles 75-130 nm in lamina I. While this type of terminal was relatively infrequent, it showed many exocytotic profiles. Two are present in the plane of sectioning (encircled).

2b. One of the exocytotic profiles at higher magnification (arrow) demonstrates a core being discharged into the extracellular space between the terminal and an axon.

2c. A second core (arrow) is being released while an adjacent large vesicle (arrowhead) is in a position that would allow fusion.

2d. A terminal in lamina I with round (75-85 nm) dense cored and many small clear vesicles in a clear cytoplasm. A core is released into the extracellular space (arrow).

2e. A large dense cored vesicle (arrow) is undergoing exocytosis at a non-synaptic site while small clear vesicles cluster and fuse with the membrane at a synapse (arrowheads).

2f. Non-synaptic exocytosis (arrow) from a large vesicle in a terminal (lamina II) with numerous small clear and a few large dense cored vesicles. The small clear vesicles undergo exocytosis at the synapse (arrowheads).
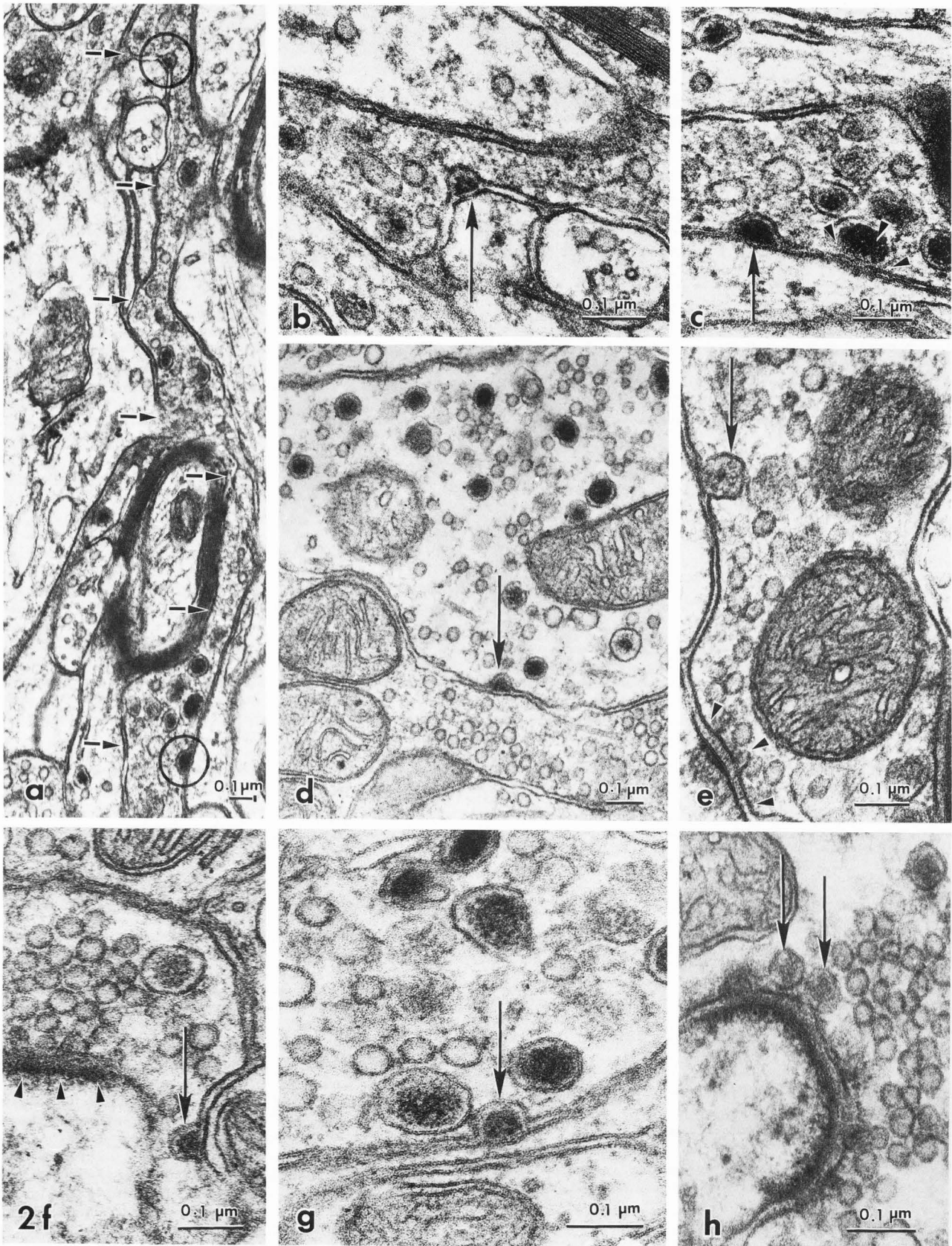
2g. Exocytosis from a large dense cored vesicle (arrow) present in a nerve terminal containing small vesicles of various sizes (40-70 nm). This type of terminal has many synapses although none is present in the micrograph.

2h. A synapse with a cluster of small and a few large dense cored vesicles (arrows) that may be in a position to undergo synaptic exocytosis. ➡

(Glazer and Basbaum 1983) associated with the vibrissae area. These may include terminals with large dense cored vesicles containing enkephalin (Bresnahan et al. 1985; Hunt et al. 1980) or substance P (Cuello et al. 1977; Del Fiacco and Cuello 1980; Priestley et al. 1982) sometimes co-stored with serotonin (Pelletier et al. 1981). In the present study the terminals in laminae I and II were studied in rats that had a lesion placed under the skin in the vibrissae area, sparing the midline and lip region. This treatment has been found to act as a potent stimulus (Zhu et al. 1981) without inflicting central degeneration (Zhu, unpublished)

Terminals with ovoid to round large dense cored vesicles, 90-130 nm

One type of slender, varicose fibers with few, if any, well defined synapses contained very large vesicles (Fig 2a) and some small clear vesicles (Figs 2b,c). Even though this category of terminal was infrequent and had few exocytotic profiles in control rats, they accounted for 30 percent of all exocytotic profiles at structurally non-specialized areas after intense stimulation. In some instances at least two examples

of exocytosis were encountered in the plane of sectioning (Figs 2a-c).

Terminals with round large dense cored vesicles, 75-85 nm

Terminals with small clear vesicles (45-55 nm) and round dense cored vesicles 75-85 nm, were relatively common in both laminae I and II. This type of terminal had some synaptic contact with other neurons. However, virtually all exocytotic release from the large vesicles occurred at non-synaptic sites (Fig 2d) even when synapses were present (Figs 2e,f).

Terminals with dense cored vesicles 80-100 nm, and small vesicles 40-70 nm

This category of terminal was common and often made synaptic contact with other neurons. Only clear vesicles appeared to be involved in the synaptic release. In contrast, the large dense cored vesicles released their contents by exocytosis at non-specialized areas of the membrane (Fig 2g). Thus, small vesicle-mediated synaptic, and large vesicle-mediated non-synaptic neurotransmission and or modulation, may co-exist, since the large vesicles are believed to store both classical transmitter and neuropeptide.

Preferential non-synaptic release from large dense cored vesicles

While the small vesicles appear to preferentially fuse with the presynaptic membrane at well defined synaptic sites, the large dense cored vesicles preferentially fuse with the membrane at non-synaptic sites. However, there were a few examples of large vesicles in the immediate vicinity or, perhaps, even fused at presynaptic membranes (Fig 2h). Nevertheless, only 1-2 percent of large vesicle exocytosis appeared to take place at specialized synaptic sites, i.e., 4 of 370 exocytotic profiles recorded in the present study.

Conclusions

(1) The combined evidence from ultrastructural, immunocytochemical and biochemical studies indicates that large dense cored vesicles in the peripheral as well as in the central nervous system store neuropeptides.

(2) Different neuropeptides may be co-stored with one of the classical transmitters, e.g., enkephalin with noradrenaline in the pig vas deferens, neuropeptide Y with noradrenaline in the rat vas deferens and vasoactive intestinal polypeptide (VIP) with acetylcholine in cat salivary gland.

(3) In the peripheral nervous system, large dense cored vesicles in noradrenergic varicosities release their contents by exocytosis along morphologically unspecialized areas of the terminal membrane.

(4) In the central nervous system, large dense cored vesicles in several different types of terminals undergo exocytosis at nonsynaptic sites even when typical synapses are present. Only rarely are large dense cored vesicles present at the structurally specialized presynaptic membrane in a position that would allow release into the synaptic gap.

(5) The co-existence of synaptic release, mainly from small transmitter-containing vesicles, and non-synaptic release from large vesicles that may contain both transmitter and neuropeptide, may have developed to serve different functions.

(6) Non-synaptic release could allow released substances to diffuse over a wider distance to interact with appropriate receptors while synaptic release would tend to restrict and direct receptor interaction.

Acknowledgements

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Discussion with Reviewers

R. Murray: The two main ideas about the dynamics of the neuropeptide vesicles in neurons are: (1) they are formed with their secretory product, presumably at the Golgi apparatus and emptied by exocytosis; (2) they are more or less permanent structures which can be loaded from and emptied to the surrounding cytoplasm through passage through the membrane of the vesicles. You show that there is an exocytosis, but do you have any information on the site of production of vesicles?

Authors: Morphological and biochemical evidence favor the first of the two concepts, i.e., that large dense cored vesicles are formed exclusively in the perikarya. These contain the organelles that are involved in synthesis and packaging of large molecules into the matrix of large vesicles, e.g., the enzyme dopamine β-hydroxylase and chromogranins in large noradrenergic vesicles. In the bovine splenic nerve, which has served as a model system for postganglionic sympathetic nerves for many years, several peptides including enkephalins, dynorphin and bombesin are also packaged into the vesicles in the cell bodies before being transported to the terminal varicosities. This has been demonstrated by taking subsequent segments between the ganglion and terminals and isolating their large vesicles prior to biochemical analyses. While the number of large molecules per vesicle does not change, its content of noradrenaline increases during the transport (see review by Klein and Thureson-Klein 1984). However, while we know that the large vesicle population is associated with neuropeptides, we do not know to what extent more than one type can be packaged into a single vesicle.

R. Murray: Is it possible to perform fluorescence microscopy and electron microscopy on the same cryostat section (Hellström, Ann Histochem 21, 15-18, 1976)? Have you tried this? What are the possible advantages of such a method? What are possible drawbacks?

Authors: We have not tried the classical formaldehyde- or more recent glyoxylic acid methods to demonstrate catecholamine fluorescence in conjunction with electron microscopy. Similarly, we have not been successful in demonstrating costorage by using the 'Faglu' method (Furness et al. 1978). Obviously, one would like to be able to identify the type of classical transmitter associated with a neuron and/ or its contents of neuropeptide(s). Unfortunately, it is difficult to maintain acceptable ultrastructural preservation in cryostat sections. Conversely, antigenicity is often lost or dislocated in tissue that has been fixed for electron microscopy. Cuello and colleagues (Cuello et al. 1982) have used the peroxidase-antiperoxidase (PAP) method in combination with radioimmunocytochemistry to localize substance P and enkephalin in the same section. We have preferred the PAP method to identify substance P or enkephalin neurons under the light microscope before sectioning for ultrastructural examination. Presently, the immunogold techniques using different size gold-particles to label co-stored substances (Varndell et al.1982) look more promising even though it is very difficult to obtain the right combination of fixation, embedding and etching to allow reliable labeling.

U. Kvist: In terminals in vas deferens the small vesicles dominate. Vas deferens is normally a resting organ in laboratory animals. Have you considered the following possibility: if small vesicles are mature forms of large vesicles, then in a resting organ the mature forms are more likely to be found than in an active organ? Consequently, have you studied proportions of large and small vesicles from sexually active animals?

Authors: We have not studied vasa deferentia from sexually active animals but have used electrically stimulated tissues from guinea pig, and others have looked at mouse (Basbaum and Heuser 1979) or rat (Pollard et al. 1982). While both large and small vesicles can be observed to undergo exocytosis when fixation takes place during stimulation (Thureson-Klein and Stjärne 1981) there is no parallel change in vesicle numbers as would be expected if small vesicles are formed from the large vesicles. While the small vesicles account for most of the noradrenaline release, there is much evidence that only the large vesicles contain soluble dopamine β-hydroxylase. Therefore, many of the small vesicles in the terminal may have been locally reformed, e.g., from internalization of terminal membrane via clathrin coated vesicles similar to the endocytosis suggested to operate in cholinergic terminals (Miller and Heuser 1984).

B. Afzelius: It is interesting that the large vesicles release their contents at unspecialized sites along the axonal or terminal membrane. In some of your figures the distance between this release site and the synapse is less than 1 µm. As diffusion of molecules within narrow clefts is very rapid, I presume that the release contents could reach the synapse within 1 msec after exocytosis. Do you think the release is related to the nerve impulse or is it a phenomenon that occurs independent of it?

Authors: Large vesicles that are proximal to the terminal membrane may fuse at structurally unspecialized areas in response to a nerve impulse as indicated by the increase in large vesicle exocytosis observed during electrical stimulation. However, with regard to release at the well defined active synaptic zone, large vesicles are rarely in a position that would allow release (Elfvin 1971). It is possible that increased synaptic activity would bring more large vesicles in position to release at the active synaptic zone as well as at the nonspecialized areas. The physiological effects would then be dependent upon the presence of appropriate receptors in the vicinity.

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