

CAPTURING AND QUANTIFYING THE EXOCYTOTIC EVENT

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Summary

Although exocytosis is now known to be the universal method by which proteins are released from eukaryotic cells, we know surprisingly little of the mechanism by which exocytosis occurs. One reason for this is that it has proved difficult to capture sufficient of these evanescent events to permit their study. The difficulty with which exocytoses can be visualized with standard preparative techniques varies among tissues, but the problem is particularly apparent in the mammalian nervous system.

Tannic acid has recently been introduced as an agent by which exocytosed granule cores can be captured and visualized electron-microscopically. Application of tannic acid to the magnocellular neurosecretory system reveals exocytoses from all parts of their terminal arborization within the neural lobe, and also from their dendrites within the hypothalamus. Quantification of the exocytoses in unstimulated tissue and in tissue stimulated by a variety of exogenous and endogenous mechanisms indicates: (a) that exocytosis occurs equally from each unit of membrane of the perivascular nerve endings, and of the axonal swellings that were previously thought to be sites of granule storage, rather than release; (b) that, in the nerve endings, a greater proportion of the stored granules are exocytosed, and thus the endings are specialized for release not by any particular property of their membrane, but by a high surface membrane:volume ratio. Together, the data cast doubt on the hypothesis that exocytosis occurs only at some functionally specialized sites at certain loci in the membrane. Rather, the data favour the hypothesis that magnocellular granules can fuse with any part of the membrane, depending on constraints imposed by the cytoskeleton, and a local increase in cytosolic free calcium level.

When applied to hypothalamic central nervous tissue, tannic acid reveals that exocytosis of dense-cored synaptic vesicles occurs preferentially, but not exclusively, at the membrane apposed to the postsynaptic element. However, about half of all exocytoses from synaptic boutons occur at bouton membrane unrelated to the synaptic cleft.

In all tissues studied, tannic acid reveals a heterogeneity among secretory cells in the extent of exocytosis that occurs in response to stimulation, and permits an analysis of the degree to which secretion is polarized in any one direction.

These results question long-held assumptions concerning the site at which neurones release transmitters and modulators. Tannic acid seems likely to prove a potent tool in the investigation of both the mechanism of exocytosis and the ways

Key words: exocytosis, neurosecretion, morphometry, tannic acid.

in which different types of cells adapt the process to perform their physiological functions.

Introduction

Exocytosis of secretory granules is currently thought to be the sole mechanism by which the 'regulated' secretion of stored proteins and peptides occurs. The mechanism appears to be common to all types of peptide-secreting cell and to all eukaryotes. 'Constitutive' secretion (Kelly, 1985) also appears to occur by exocytosis, though in this case the secreted products are not stored; plasma membrane proteins also reach their destination by exocytosis.

Proteins destined for secretion are unable to diffuse through the plasma membrane; they cross lipid bilayers only when they are transported through the membrane of the rough endoplasmic reticulum as they are translated. Thereafter, the proteins are transferred to the Golgi apparatus and then to the cell surface by a series of membrane-membrane fusion events of which the final one is exocytosis. Amine hormones and neurotransmitters, though much smaller and able to diffuse across membranes, are concentrated into secretory granules in the chromaffin cells of the adrenal medulla, in aminergic neurones (particularly their terminals) and in mast cells. The amines are released, often with co-packaged peptides, by exocytosis.

Compelling evidence for exocytosis as the method of release comes from two sources: electron microscopic visualization of the event and demonstration, by biochemical techniques, that substances of very different physical properties, which are co-stored in a granule, are also co-released. Evidence of such stoichiometry is available for a number of mammalian systems. The earliest evidence for co-secretion of granule components was the concomitant release of ATP and amines from the adrenal medulla (Carlsson, Hillarp & Hökfelt, 1957). More rigorous data were later provided by Douglas & Poisner (1966), who suggested that their results were compatible with release by exocytosis. It is now clear that chromaffin cells co-release appropriate ratios of amines, nucleotides, chromogranins, enkephalins and enzymes (Viveros, Diliberto, Hazum & Chang; 1979; Livett *et al.* 1981; Hook & Eiden, 1985). Dopamine β -hydroxylase is also co-released with amines from splenic nerves (Smith, 1971; Dixon, Garcia & Kirpekar, 1975). Close parallelism between released posterior pituitary hormones and the neurophysins that arise from the same precursor has been demonstrated (Utten-thal, Livett & Hope, 1971; Nordmann, Dreifuss & Legros, 1971), and the same principle applies for a number of other tissues.

Ultrastructural evidence for the release of peptide-containing secretory granules from endocrine cells was first presented by De Robertis & Vaz Ferreira (1957). Comparable evidence for exocytotic release of neurosecretory granules (from neurosecretory neurones of the earthworm cerebral ganglion) was not published until 5 years later (Rolich, Aros & Vigh, 1962), though these authors did not appear to appreciate the significance of the observations. This was left to Normann

(1965), who described exocytotic release of granules from extrinsic neurosecretory neurones and intrinsic paraneurones in the locust corpus cardiacum, an analogue of the vertebrate posterior pituitary.

In spite of these early observations in invertebrate systems, exocytosis in other, particularly mammalian, tissues proved difficult to visualize. The likely reasons are that chemical fixatives take a finite time to immobilize granule cores, which probably disperse very rapidly after release in mammals (in insect systems, dispersal of granule cores may occur less rapidly than in vertebrates, Scharrer & Wurtzelmann, 1978), and that even if all exocytoses occurring at the moment of fixation were captured, their expected frequency is low (Morris, 1976; Normann, 1976). Such difficulties prompted Douglas, Nagasawa & Schulz (1971), when describing exocytosis in the posterior pituitary, to remark 'All this suggested to us that the failure to demonstrate exocytosis in the neurohypophysis ... might simply reflect the difficulty of capturing such doubtless fleeting events'.

In the absence of clear images of exocytosis of granules in tissues such as the posterior pituitary, exocytosis was inferred from the demonstration of a reduction in the number of granules present. In the posterior pituitary, the neurosecretory axons become dilated in places to form nerve endings, which abut the perivascular basal lamina, and other, preterminal nerve 'swellings' which lie more deeply in the lobules. Stimulation of release by $56 \text{ mmol l}^{-1} \text{ K}^+$ results in a detectable reduction in the number of granules only in nerve endings, and not in nerve swellings or undilated axons (Nordmann & Morris, 1976). This was interpreted both as evidence for exocytosis, and that exocytosis was restricted to the nerve endings near their contact with perivascular basal lamina. However, this approach cannot easily take into account migration of granules within the tissue, and must necessarily favour detection of release in sites where a large proportion of total granules are released, and discriminate against detection of the release of small numbers of granules from compartments with numerous granules.

Although there are now published images of exocytosis from most secreting tissues, exocytosed cores were more easily visualized in some mammalian endocrine tissues, such as the adrenal, and in some species, such as the hamster (Diner, 1967; Grynszpan-Winograd, 1969; Nagasawa, Douglas & Schulz, 1970; Benedeczky & Smith, 1972). It is easy to see that, if the rate of secretion is particularly high, or the granule cores take a relatively long time to disperse, the frequency with which exocytoses can be seen would vary among tissues. It is much less easy to understand why there should be variation amongst mammals.

Freeze-fracture has been used to capture exocytoses in a variety of tissues. In the posterior pituitary it appears to be more efficient in this respect than chemical fixation (Santolaya, Bridges & Lederis, 1972; Theodosis, Dreifuss & Orci, 1979); the same is true of the crab sinus gland (Andrew & Shivers, 1976; Shivers, 1976). Freeze-fracture reveals membrane proteins as intramembranous particles (IMPs). In unstimulated preparations of isolated granules from islets of Langerhans, the IMPs are randomly distributed over the concave A fracture face rather than the convex B fracture face but, during stimulation, particle-rich areas appear in

the B face at sites of fusion of granules (Dahl & Gratzl, 1976). In contrast, Orci, Perrelet & Friend (1977) report fusion of particle-deficient granule membranes, implying fusion of lipid bilayers alone. Freeze-fracture combined with ultra-rapid freezing has been used to provide a particularly elegant demonstration of the site of exocytosis of synaptic vesicles at the neuromuscular junction of frog cutaneous pectoris muscle and correlation of the exocytotic event with quantal transmitter release (Heuser *et al.* 1979). It has also revealed rather small exocytotic pores in secreting mast cells, whereas transmission microscopy shows wide-mouthed omega figures (Chandler & Heuser, 1980). Most tissues, however, are not physically suited to the investigation of exocytosis by rapid freezing techniques.

The mechanism of exocytosis

For such a fundamental and omnipresent process, we know surprisingly little of the events involved in the membrane fusion leading to exocytosis (Burgoyne & Cheek, 1987) and multiple controls may be involved in this calcium-dependent event (Baker, 1984). Exocytosis consists of a number of morphologically definable steps. The initial step involves the connection of the granule membrane with the plasmalemma; this process may involve fine 'connecting strands' (Benedeczky & Smith, 1972; Normann, 1976), granule-associated pseudopodial structures (Castel, 1977; Scharrer & Wurtzelmann, 1977) (see Figs 2B, 13) or, in the case of casein granules, an adhesion plaque (Franke *et al.* 1976). The granule and plasma membranes then fuse, forming a transient pentalaminar membrane (Douglas, 1968; Tanaka, De Camilli & Meldolesi, 1980) and a fusion pore that initially has characteristics similar to that of an ion channel before increasing in conductance (presumably as the pore enlarges; Breckenridge & Almers, 1987). The granule core is thereby exposed to the exterior of the cell, resulting in the electron microscopic appearance of a classical 'omega' profile (see Meldolesi, Borgese, De Camilli & Ceccarelli, 1978).

Granules can fuse, not only with the plasmalemma, but also with other granules to form compound exocytotic figures (Phillips, Burrige, Wilson & Kirshner, 1983) (see Figs 2B, 6, 7). Compound exocytosis seems to occur particularly when the stimulus to release is very potent, and in certain cell types, such as mast cells, corticotrophs and thyrotrophs: e.g. stimulation of mast cells with calcium ionophores (Kagayama & Douglas, 1974); stimulation of the anterior pituitary by large doses of TRH (Okino *et al.* 1979), by a potent analogue of cyclic AMP (Pelletier, LeMay, Béraud & Labrie, 1972), by the calcium ionophore A23187 (Fujita, Kurihara & Miyagawa, 1983), and by the phospholipase A₂-activators mellitin and mastoparan (Kurihara *et al.* 1986); stimulation of the sinus gland of the crab with $56 \text{ mmol l}^{-1} \text{ K}^+$ (Nordmann & Morris, 1980). Fusion of isolated neurosecretory granules *in vitro* can be induced by varying concentrations of calcium and other divalent ions (Dahl & Gratzl, 1976; Gratzl, Dahl, Russell & Thorn, 1977). Vesicles formed from pure phospholipids can fuse *in vitro* only after a significant energy barrier, which probably results from the need to remove tightly

bound water, is overcome (Linstedt & Kelly, 1987). Therefore, the ability of granules and plasma membranes to fuse may not depend on the presence of specific components localized to special regions of the plasmalemma. Equally, a search for exocytosis-specific proteins in granule membranes has not proved successful (Rubin, Lyubkin & Pressman, 1984). On the positive side, we now know considerably more about possible controlling interactions between secretory vesicles and the cytoskeleton *via* such proteins as synapsin I, caldesmon and fodrin (see Linstedt & Kelly, 1987; Aunis & Bader, 1988) and synexin (Pollard, Burns & Rojas, 1988).

What is the duration of an exocytotic event?

We have very little information on this point which is of considerable importance in the interpretation of the number of exocytoses seen. Douglas (1974) used high-speed cinematography to time exocytosis from mast cells stimulated by the polyamine 48-80 and concluded that granule expulsion is completed within an upper limit of 6–8 ms. Roughly similar conclusions have been reached from a study of exocytosis-related changes in capacitance (2–59 ms) of mast cells from beige mice (which have abnormally large granules) (Breckenridge & Almers, 1987; Zimmerberg, Curran, Cohen & Brodwick, 1987), and from rapid-freezing studies of cholinergic vesicle release (2–4 ms) at frog neuromuscular junctions (Heuser *et al.* 1979).

It is not possible within this time frame to assay the appearance of peptide released from cells in the media which surrounds them. However, if the granule cores remain intact rather than dispersing, then their contents can be defined by immunocytochemistry (Li, Dubois & Dubois, 1979).

For most tissues, neither standard transmission nor freeze-fracture electron microscopy have provided consistent large numbers of images of exocytosis that would permit analysis either of the anatomical distribution of exocytosis around the cell, or of the number of exocytoses in unstimulated and physiologically stimulated tissues.

What is needed is a technique capable of capturing all, or at least a definable proportion of, exocytoses over an appropriate period. Such a technique must not itself induce exocytosis and should be compatible with both immunocytochemical identification of the captured cores and physiological manipulation of the tissue. With such a technique the following questions can be addressed. (1) From which cells in a population does release occur? What is the extent of secretory heterogeneity in the tissue? Does this change with stimulation? (2) Where in the cell does exocytosis occur? Some cells (e.g. mast cells) are thought of as unpolarized with respect to their secretory function; at the other end of the scale the neurone is thought to be extremely polarized and to release neurotransmitter from axonal terminals only.

■ If exocytosis were limited to one site in the cell, this could help to focus experimental attack on the fundamental mechanisms of exocytosis.

Tannic acid

Tannic acid is a complex 1701 Da polymer of gallic acid and glucose residues which binds to amino and carboxyl groups of extracellular proteins, but is incapable of penetrating the plasmalemma of unfixed tissues, or tight junctional structures such as those forming the vertebrate blood–brain barrier, or comparable structures in invertebrates. When tissues are exposed to tannic acid, either combined with aldehyde fixatives or included in physiological saline, proteinaceous secretory granule cores are mordanted as they are extruded, and can thereby be visualized by electron microscopy. Tissues appear to survive well in physiological solutions containing tannic acid and the extruded cores accumulate as exocytosis continues. However, one caveat is that tannic acid can depolarize frog sartorius muscle, apparently by an action on chloride channels (Gladwell, Bowler & Duncan, 1971).

Exocytosis in invertebrates revealed by tannic acid

This technique, pioneered by Buma, Roubos and co-workers, found its first application in invertebrate nervous and endocrine systems, particularly in the snail *Lymnaea stagnalis*, where the number of exocytotic figures in a defined population of peptidergic neurones has been shown to reflect the assayable secretory activity of these neurones in response to secretagogues such as cyclic AMP analogues (Roubos & Van der Wal-Divendal, 1980; Buma & Roubos, 1984, 1985*a,b*; Buma, Roubos & Brunnekreef, 1986).

In conventionally fixed locust corpora cardiaca, exocytosis of large (200–400 nm diameter) granules from the intrinsic neurones is readily demonstrable (Fig. 1A) at rest, and particularly when stimulated by flight; in contrast, exocytosis of small

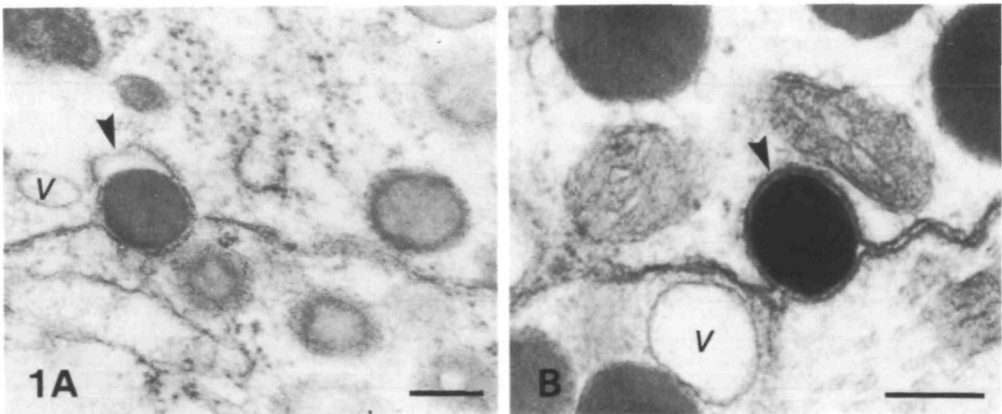


Fig. 1. Exocytosis (arrowhead) of a secretory granule from an intrinsic neurone in the corpus cardiacum of a locust which has been conventionally fixed (A), or fixed in medium containing tannic acid (B). Tannic acid confers enhanced electron density on the extruded granule core but does not influence other aspects of cytology such as the presence of vacuoles (v). Scale bar, 200 nm.

(100 nm diameter) granules from the octopaminergic secretomotor neurones innervating the intrinsic neurones is not evident (Rademakers & Beenackers, 1977). Injection of tannic acid (in either an aldehyde fixative or a physiological saline), however, captures granule exocytosis both from the intrinsic neurones (Fig. 1B) and from the secretomotor neurones. Morphometric analysis reveals that the site of granule release from the intrinsic neurones remains unaltered by tannic acid treatment, but that the numbers of exocytotic profiles are dramatically increased (Pow & Golding, 1987).

Exocytosis in vertebrates revealed by tannic acid

Buma and his colleagues have more recently applied tannic acid to a variety of vertebrate secretory tissues. They have demonstrated that, in the magnocellular neurosecretory system, exocytosis is not confined to the nerve endings as had been thought, but occurs from endings, swellings and undilated axons in the neural lobe and from the portions of the magnocellular axons in the median eminence (Buma, Roubos & Buijs, 1984; Buma & Nieuwenhuys, 1987, 1988; Van Putten, Killian & Buma, 1987). These observations suggest that the concept of the neurone releasing only from its synaptic bouton-like nerve ending must be radically reappraised for magnocellular neurones at least. Further, they provide evidence that the release of vasopressin by magnocellular axons in the median eminence occurs by exocytosis (Holmes, Antoni, Aguilera & Catt, 1986). What these studies do not provide, however, is a quantitative analysis of the exocytoses that had been captured, nor were the experiments able to address the question of release of peptides within the central nervous system.

Heterogeneity of release among cells

Tannic acid allows ready identification of numerous exocytoses of chromaffin granules (Fig. 2B) (Golding & Pow, 1987). Exocytosis occurs all round chromaffin cells and not only from their vascular poles (Grynszpan-Winograd, 1969). When the exocytoses from randomly oriented cell profiles are quantified (Fig. 2A), the distribution of exocytoses among cells is clearly heterogeneous, many cells releasing a few, but other cells releasing many granules. Exocytosis from anterior pituitary gonadotrophs is also not polarized (C. E. Lewis & J. F. Morris, unpublished observations). In the neural lobe, the terminal arborizations of magnocellular neurones also show a very variable number of exocytoses from one process to another (see Fig. 6).

Release of granules by exocytosis from magnocellular neurones

Where does exocytosis occur?

Our observations on the terminal arborization of magnocellular neurones confirm and extend those of Buma and his colleagues. Exocytosis of magnocellular granules can be visualized at all parts of the membrane of nerve endings (Fig. 3)

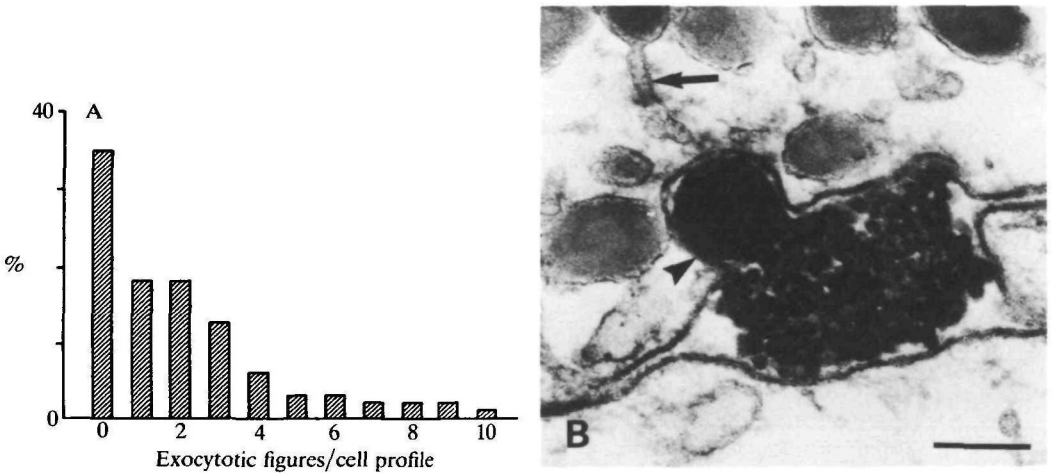


Fig. 2. (A) Histogram showing frequency of granule exocytotic profiles at the surface of each of 100 sequentially encountered sectioned profiles of rat adrenal medullary adrenergic chromaffin cells. (B) Compound exocytotic release of adrenergic chromaffin granules from the frog adrenal gland (arrowhead). Note the increased density of the tannic-acid-captured core, and the dispersion into smaller subunits of part of the extruded cores. One granule bears an elongated pseudopodial structure (arrow) which might play a role in exocytosis. Scale bar, 200 nm.

and also from nerve swellings (Figs 4, 6), often in apposition to pituicytes (Fig. 7), and from undilated axons (Fig. 5). In Brattleboro rats which lack large (160 nm diameter) vasopressin-containing granules, exocytosis of the small (100 nm diameter) granules contained in the mutant neurones can be visualized and readily distinguished (Morris, Pow & Shaw, 1988).

The dendrites of magnocellular neurones also contain considerable numbers of neurosecretory granules, the fate of which has thus far been quite unclear. Modification of the tannic acid technique for application to brain slices, to circumvent the blood-brain barrier, readily demonstrates release of magnocellular granules by exocytosis from dendrites in the ventral glial lamina of the

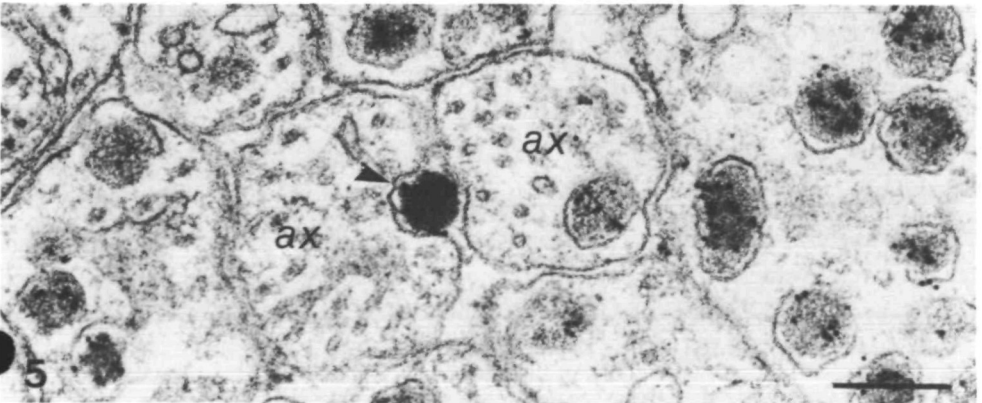
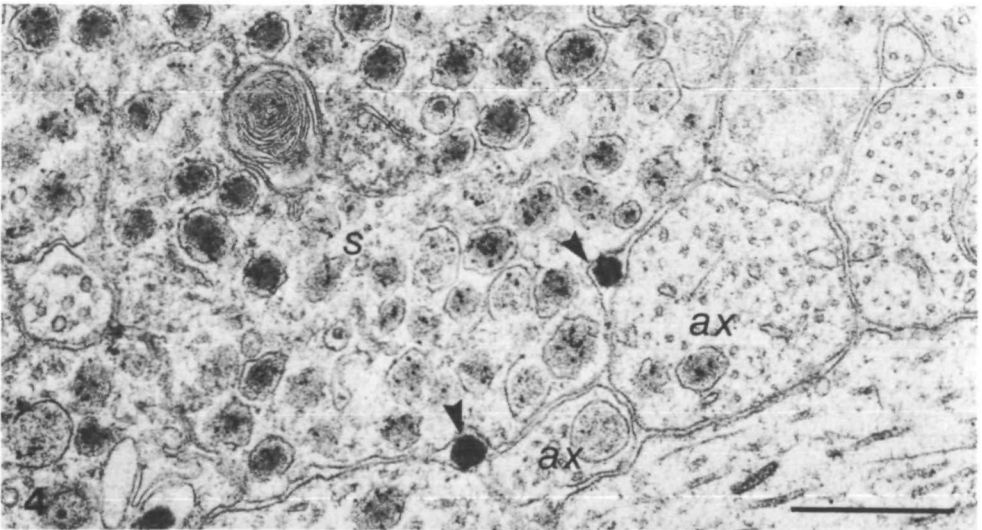
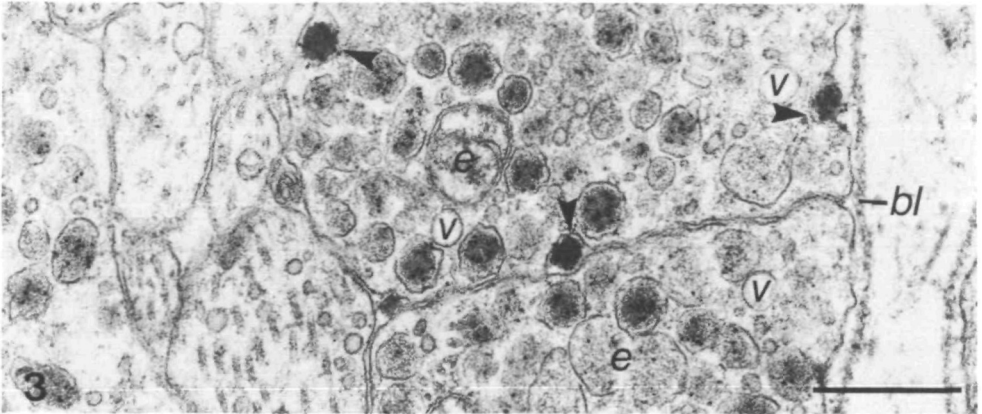
Figs 3–5. Rats perfused with tannic-acid-containing saline prior to fixation.

Fig. 3. Exocytosis of immunogold-labelled oxytocin-containing granules (arrowheads) from nerve ending (*e*) in the posterior pituitary. Granules are released both sites in apposition to the pericapillary basal lamina (*bl*), and at sites distal to it. Lucent vacuoles (*v*), which may represent part of the endocytotic pathway for granule membranes, and smaller microvesicles are also distributed throughout the ending. Scale bar, 0.5 μ m.

Fig. 4. Exocytosis of immunogold-labelled oxytocin granules (arrowheads) from a nerve swelling (*s*) in the posterior pituitary. In this example granules are being extruded in apposition to preterminal undilated magnocellular axons (*ax*). Scale bar, 0.5 μ m.

Fig. 5. Exocytosis of an immunogold-labelled vasopressin granule (arrowhead) from an undilated preterminal axon (*ax*) in the posterior pituitary. Scale bar, 200 nm.

supraoptic nucleus in tissue stimulated with barium (Figs 8, 9, 10). Exocytosis from dendrites could well account for a large part of the release of oxytocin and vasopressin into the hypothalamus and cerebrospinal fluid (Robinson, 1983; Moos



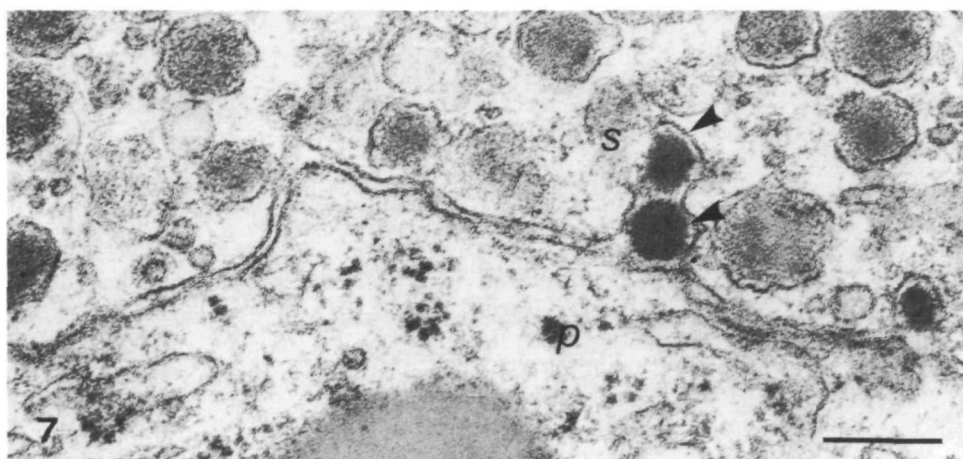
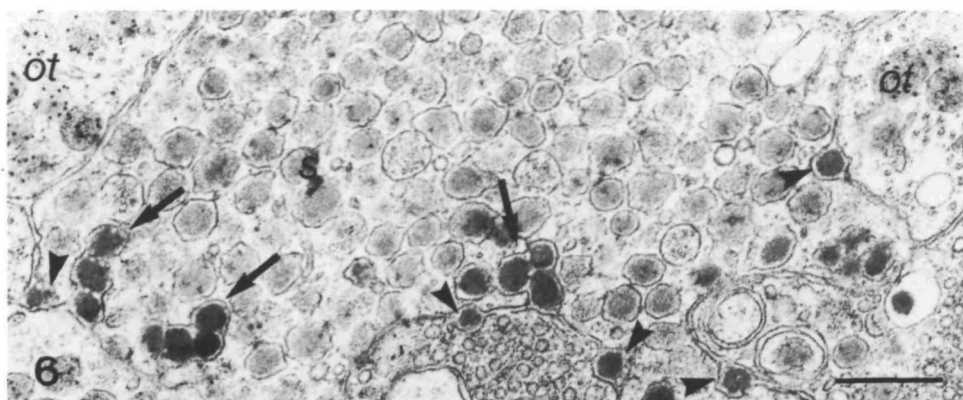


Fig. 6. Rats perfused with tannic-acid-containing saline prior to fixation. Ultrathin section of tannic-acid-treated posterior pituitary, immunogold-labelled for oxytocin-neurophysin. Oxytocin-containing profiles (*ot*) are labelled whereas the large vasopressin-containing swelling (*s*) contains unlabelled secretory granules. Some unlabelled vasopressin-containing granules have formed compound (arrows) and single (arrowheads) exocytotic figures. Scale bar, $0.5\ \mu\text{m}$.

Fig. 7. Pituitary from a rat which has been perfused with tannic-acid-containing fixative, showing compound exocytosis of two vasopressin-containing granules (arrowheads) in apposition to a pituicyte (*p*) identified by the presence of a lipid droplet. Scale bar, $200\ \text{nm}$.

et al. 1984; Mason *et al.* 1986), though the elucidation of the relative contribution of release from dendrites and from synaptic boutons containing the neurohormones (e.g. Buijs & Swaab, 1979; Theodosis, 1985) must await further experiment. The method opens the possibility of determining the site of release very precisely and this may well be important, in view of the known effects of oxytocin on the behaviour of oxytocin cells during the milk ejection reflex (Freund-Mercier & Richard, 1984) and on the morphological plasticity of the hypothalamic neurohypophyseal system (Theodosis *et al.* 1986b).

Table 1. Incidence of tannic-acid-captured exocytosis, and its distribution among nerve endings, nerve swellings and undilated axons in the neural lobe of unstimulated animals and animals stimulated to release oxytocin and vasopressin by a variety of exogenous and endogenous stimuli

Experimental group	Exocytoses/ $10^4 \mu\text{m}^2$ section				Distribution (%)			
	Oxytocin	Vasopressin	Total	Ending	Swelling	Axon	?	
Long Evans rats								
Unstimulated	4	12	16	53	36	1	10	
$56 \text{ mmol l}^{-1} \text{ K}^+$	45	148	193	51	38	2	9	
$56 \text{ mmol l}^{-1} \text{ K}^+ + 1 \text{ mmol l}^{-1} \text{ EGTA}$	—	—	29	—	—	—	—	
Naloxone-treated, morphine-dependent	36	7	43	49	46	1	4	
Mice								
Unstimulated	4	39	43	—	—	—	—	
<i>di/di</i> nephrogenic diabetes insipidus	9	65	74	—	—	—	—	

— not determined; ? origin of exocytosed granule in doubt.

Mean values determined from examination of multiple grid squares in more than one animal.

Does tannic acid reveal 'physiological' exocytosis?

There is no evidence that exposure to tannic acid induces exocytosis, and quantitative analysis of the number of exocytoses in different conditions (Table 1) suggests that such treatment does not interfere with 'physiological' release of vasopressin or oxytocin. Thus, the number of exocytoses is increased by stimulation with $56 \text{ mmol l}^{-1} \text{ K}^+$, in a calcium-dependent manner; exocytosis of oxytocin-containing granules predominates in the naloxone-treated, morphine-dependent rat in which preferential release of oxytocin can be demonstrated *in vivo* by assay (Bicknell, Leng, Lincoln & Russell, 1984); and, as expected, vasopressin secretion predominates in mice with nephrogenic diabetes insipidus.

From consideration of the number of exocytoses visualized and of the amount of hormone released, we calculate that tannic acid probably captures all the exocytoses that occur (Morris *et al.* 1988).

Are parts of the neuronal membrane specialized for release?

Since exocytosis from magnocellular neurones is in no way topographically restricted to the nerve endings, which had previously been considered to be the neurohaemal release site, we must ask whether the nerve ending is in any way specialized for release. Calculation of the ratio between the amount of membrane of nerve endings and nerve swellings, and the number of exocytoses visualized at the surface of each of these compartments, reveals that the numbers of exocytoses per unit membrane of nerve ending and nerve swelling are almost identical (Morris *et al.* 1988). The same conclusion is reached from experiments in which hormone release from fractions of neurosecretosomes enriched in either nerve endings or nerve swellings is assayed (Nordmann *et al.* 1988). It would therefore appear that the membrane of these two compartments does not differ in its ability to interact with the granules to produce exocytosis. We do not yet have similar calculations for the membrane of axons and dendrites, but it is possible that, in magnocellular neurones, no part of the membrane is particularly specialized for release. Were that to be true, then the localization of release in magnocellular neurones must be determined by the availability of granules to the membrane. The cytoskeleton would be expected to play a major role in determining this (Aunis & Bader, 1988), but local control of calcium fluxes might well also be important.

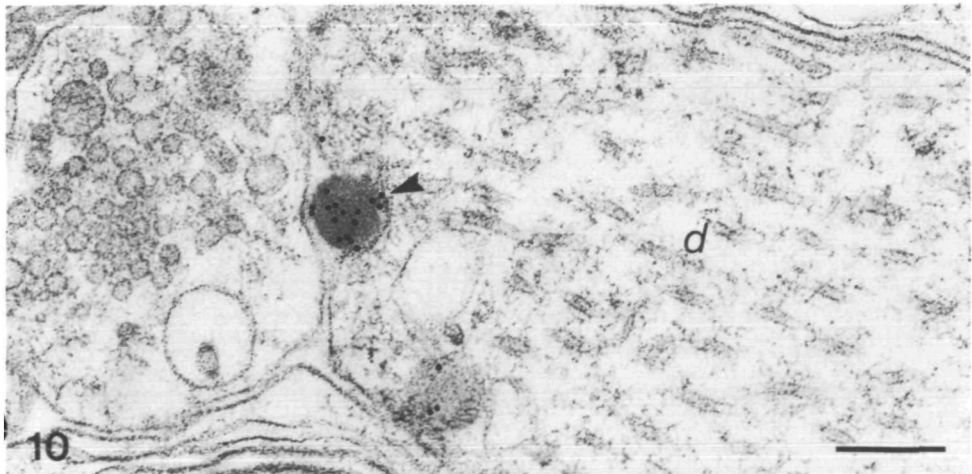
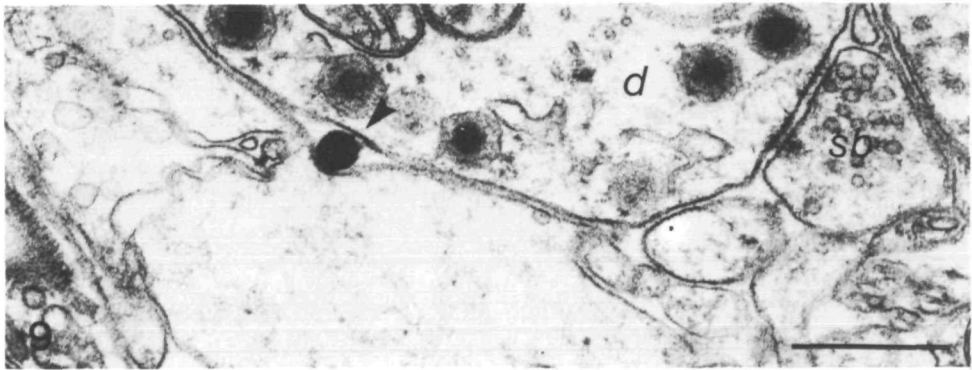
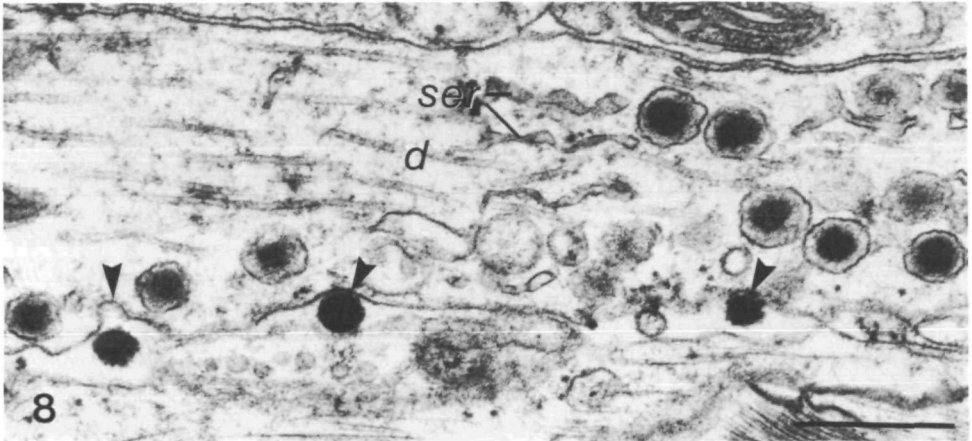
Figs 8–10. Rat hypothalamic brain slices, incubated in tannic-acid-containing modified Yamamoto's media for 10 min, prior to fixation.

Fig. 8. Exocytosis of secretory granules (arrowheads) from a magnocellular dendrite (*d*) in the ventral glial lamina below the supraoptic nucleus. The dendrite contains abundant smooth endoplasmic reticulum (*ser*) and some ribosomes. Scale bar, $0.5 \mu\text{m}$.

Fig. 9. Exocytosis of a secretory granule (arrowhead) from a magnocellular neurone in the ventral glial lamina. A synaptic bouton (*sb*) contacts the dendrite (*d*). Scale bar, $0.5 \mu\text{m}$.

Fig. 10. Exocytosis of an immunogold-labelled vasopressin granule (arrowhead) from a magnocellular dendrite (*d*) in the ventral glial lamina. The core is extruded onto an adjacent synaptic bouton. Scale bar, 200 nm .

The finding that exocytosis can occur from most parts of magnocellular neurones does not, however, mean that the nerve ending is not specialized for release. It abuts the perivascular basal lamina and thus hormone released from the nerve endings will have almost immediate access to the bloodstream. Furthermore, the ratio between the number of granules exocytosed and the number of



granules in the compartment is threefold higher for nerve endings than for the much larger nerve swellings (Morris *et al.* 1988). Thus the turnover of granules by exocytosis is threefold higher in the nerve endings than in the nerve swellings. The nerve ending is therefore specialized both by its position and by its higher surface:volume ratio. This is entirely consistent with the preferential release of newly formed hormone (Sachs & Haller, 1968) and the accumulation of newly formed granules in the nerve endings (Heap, Jones, Morris & Pickering, 1975).

Exocytosis of magnocellular granules from nerve swellings abutting pituicytes might serve a particular physiological purpose. The magnocellular granules contain opioid peptides; pituicytes have receptors for opioids and part of their control of neurosecretion from the nerve terminals could be exerted by this route (see Hatton, 1988; Bicknell, 1988). Likewise, release of magnocellular granules from dendrites in the hypothalamus would bring these peptides into direct contact with magnocellular neurones, and with the astrocytes which surround them and which show marked changes in the extent of their coverage of the magnocellular neurones in different functional states (Theodosis *et al.* 1986a; Chapman *et al.* 1986).

Exocytosis of dense-cored vesicles from other hypothalamic neurones

We have used the tannic-acid-treated hypothalamic slice preparation to investigate the exocytosis of dense-cored vesicles from the boutons of intrinsic hypothalamic neurones in the region just dorsal to the supraoptic nucleus. Incubation of unstimulated hypothalamic slices in tannic-acid-containing solutions reveals very few exocytoses, but if the slices are depolarized by application of either $56 \text{ mmol l}^{-1} \text{ K}^+$ or the calcium ionophore A23187, abundant exocytoses of the electron-dense cores of approx. 100 nm diameter dense-cored vesicles can be visualized (Figs 11, 12). The exocytoses are located not only at the regions of synaptic thickening ('synaptic' sites; Fig. 11), but also in the cleft between the bouton and postsynaptic element at regions lacking synaptic membrane thickening ('parasynaptic' sites; Figs 11, 12) and also in regions of the membrane unrelated to the postsynaptic element ('unspecialized' sites; Figs 11, 12) and even in undilated unmyelinated axons.

When the frequency of exocytosis at these various sites is considered in relation

Figs 11, 12. Rat hypothalamic brain slices, incubated in tannic-acid-containing modified Yamamoto's media for 10 min, prior to fixation.

Fig. 11. Exocytosis of secretory granules (arrowheads) from a synaptic bouton (*sb*), in apposition to an adjacent dendrite (*d*) and synaptic bouton. Exocytoses occur at 'synaptic' (*s*) sites opposed to postsynaptic densities (arrows), from 'parasynaptic' (*p*), and from apparently 'unspecialized' sites (*u*) of the bouton membrane. Scale bar, $0.5 \mu\text{m}$.

Fig. 12. Exocytosis of secretory granules from a synaptic bouton (*sb*) in the rat hypothalamus. Granules are exocytosing from 'parasynaptic' sites (*p*) lateral to the synaptic density (*sd*), and from morphologically 'unspecialized' sites (*u*) not in apparent contact with the postsynaptic element. Note the coating of the membrane of the 'parasynaptic' exocytosis. Scale bar, $0.5 \mu\text{m}$.

to the amount of membrane (Table 2), a number of interesting points emerge. The incidence of exocytosis is very similar for both the synaptic and parasynaptic areas of membrane but exocytoses are six times less frequent at membrane not apposed to the postsynaptic element. However, in this sample at least, membrane apposed

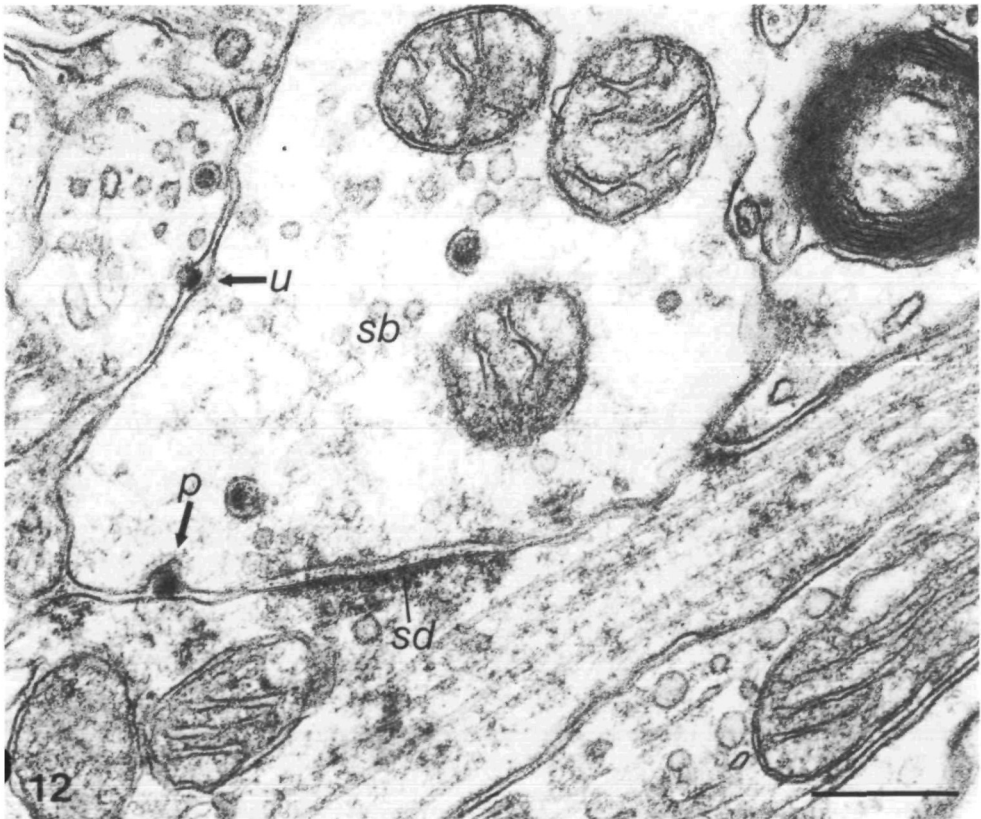
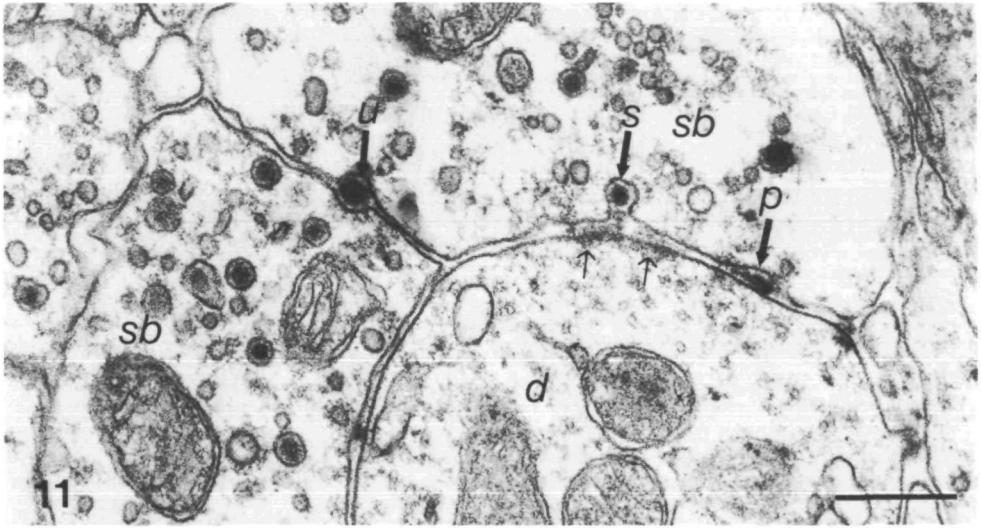


Table 2. *Distribution of exocytoses of dense-cored granules into the synaptic cleft (synaptic), adjacent to the synaptic cleft (parasympaptic), and from other unspecialized regions of synaptic bouton membrane not opposed to the postsynaptic element*

Site	Unstimulated	Stimulated	
		56 mmol l ⁻¹ K ⁺	A-23187
Synaptic			
Length of membrane (μ m)	—	21	17
Exocytoses	—	26	19
Exocytoses/ μ m membrane		1.2	1.1
Parasympaptic			
Length of membrane (μ m)	—	39	38
Exocytoses	—	35	48
Exocytoses/ μ m membrane		0.9	1.3
Unspecialized			
Length of membrane (μ m)	—	325	354
Exocytoses	—	61	69
Exocytoses/ μ m membrane		0.2	0.2

Data determined from 100 sequentially encountered bouton profiles showing one or more exocytoses.

— too few to assess.

to the postsynaptic element comprised only about one-sixth of the total bouton membrane, and therefore a similar amount of neurosecretory vesicle content is released against the postsynaptic element and at apparently unspecialized sites distant from the synaptic cleft. The incidence of exocytosis from undilated axons has yet to be quantified, but is probably much lower.

These observations lead to two conclusions. First, the membrane opposing the postsynaptic element could be intrinsically specialized for exocytosis or, alternatively, exocytosis could occur preferentially at this membrane because granules in the boutons are partially restricted to contact with membrane of this region. Second, about half of the neurotransmitter(s) contained in dense-cored vesicles is released at regions quite distant from the postsynaptic sites apparently specialized for their reception. The localization of transmitters and their receptors does not always coincide. A more widespread release of neurotransmitter than had been anticipated could, in part, provide some rationale for this discrepancy. Also, the longer term neuromodulatory actions of neuropeptides (e.g. Jan & Jan, 1982) may in part be explained by their more diffuse release.

Exocytosis from apparently unspecialized parts of axons and from dendrites is not restricted to magnocellular neurones. Exocytosis of large dense-cored amine granules at morphologically unspecialized membrane in peripheral aminergic neurones (Thureson-Klein, 1983), and in axons and dendrites of the neuropile of the trigeminal nucleus caudalis (Zhu, Thureson-Klein & Klein, 1986) can be visualized, and such observations complement the demonstration by assay of the

release of acetylcholine from preganglionic sympathetic axons (Vizi, Gyires, Somogyi & Ungváry, 1983).

Association of coated vesicles with exocytosed granule cores

Retrieval of the membrane of granules must follow their exocytosis. Coated vesicles are frequently seen in association with tannic-acid-arrested exocytoses (Figs 13, 14). This is not attributable to the tannic acid but has also been a consistent observation for exocytoses in conventionally fixed material (Fig. 1A) (e.g. Benedeczy & Smith, 1972; Douglas, 1974; Rademakers & Beenackers, 1977). The association has led to the hypothesis that the coated vesicles retrieve the membrane of the granules that have been exocytosed (Douglas, 1984).

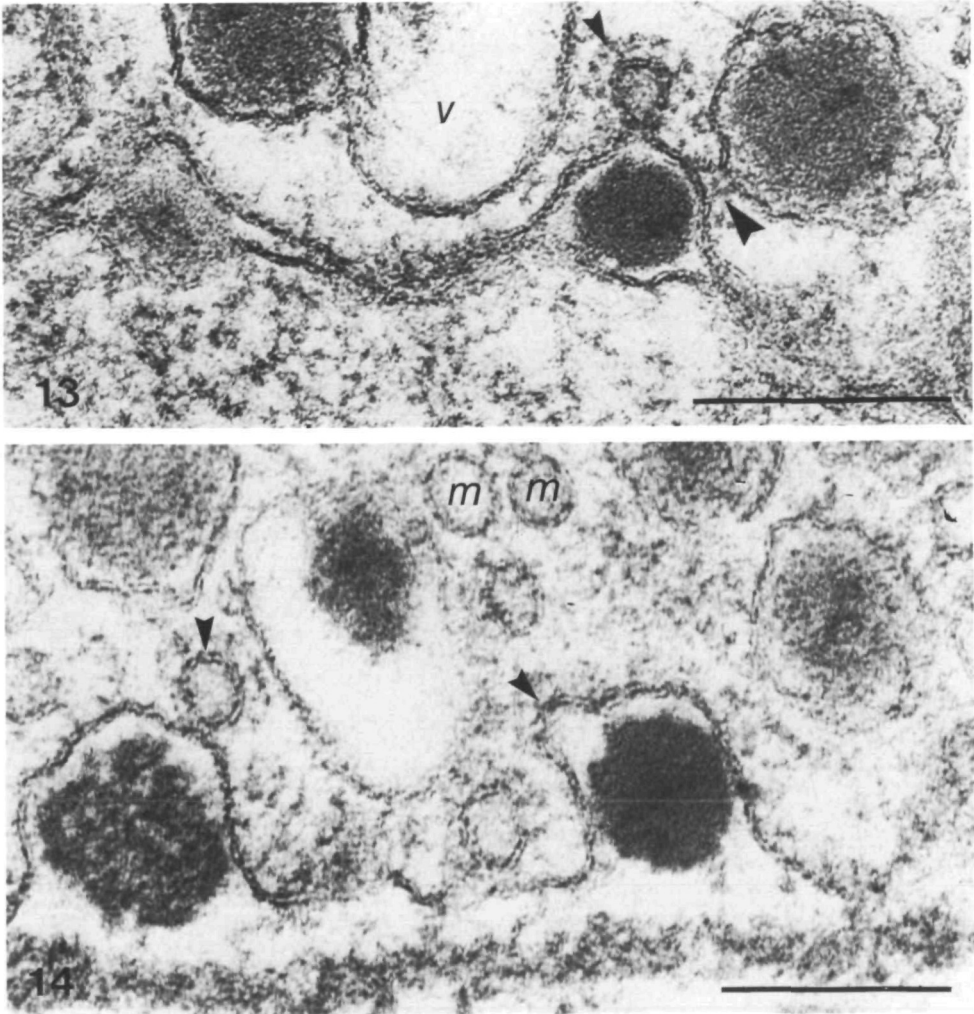
In the magnocellular system, there is no unequivocal evidence that coated vesicles recapture granule membrane; indeed, large vacuoles the size of granules appear to be the recapture organelle (Morris & Nordmann, 1980; Morris, Nordmann & Shaw, 1982). Coated vesicles in the magnocellular nerve endings could represent the retrieved membrane of the microvesicles that characterize the nerve endings and appear to have a primary function in calcium homeostasis (see Morris *et al.* 1982). As yet no marker for magnocellular granule or microvesicle membrane has been followed between compartments by electron microscopy.

The enzyme dopamine β -hydroxylase (DBH) is partly located in the membrane of chromaffin granule membranes and can serve as a marker of their fate. Stimulation of chromaffin cells incubated with fluorescently labelled antibodies to DBH results in the transient appearance on the cell surface of small fluorescent patches, which are subsequently internalized in a non-dispersed form (Phillips *et al.* 1983), shown by electron microscopy to be coated vesicles (Patzak & Winkler, 1986). If the membrane as a whole is handled in the same way as the DBH, then membranes of chromaffin granules fuse transiently with the plasmalemma, and are subsequently endocytosed by coated vesicles within a few minutes.

Conclusions

Exocytosis is a fundamental process by which cells release protein and amines, but many aspects of the process are poorly understood. Tannic acid provides a powerful tool for the capture of exocytotic events. Exposure to tannic acid does not appear significantly to affect physiological release of hormones and neuropeptides, which can be stimulated in its presence.

Release of neuropeptide by exocytosis is shown to occur not only from all parts of the terminal arborization of magnocellular neurones, but also from their dendrites. For these cells, therefore, the concept of the neurone as a uniquely polarized secretory cell requires radical revision. Quantification of the incidence of exocytosis reveals that exocytosis is equally likely to occur from each unit of membrane of the nerve swellings and the nerve endings, and also occurs from undilated axons. This calls into question the hypothesis that there are specific sites



Figs 13, 14. Posterior pituitary of a rat perfused with tannic-acid-containing saline prior to fixation.

Fig. 13. A granule exocytotic profile is surmounted by a coated vesicle (small arrowhead); large vacuole (*v*). A membranous 'neck' structure (large arrowhead) links an adjacent granule to the exocytosis. Scale bar, 200 nm.

Fig. 14. Detail of a neurosecretory nerve ending, showing two exocytosing granule cores (arrowheads), each surmounted by coated structures in varying stages of formation, and of a similar size to microvesicles (*m*) which characterize the nerve ending. Scale bar, 200 nm.

in the terminal arborization at which exocytosis can occur. It would, however, be compatible with models in which exocytosis depends either on the ability of granules to reach the plasmalemma, possibly determined by their release from the cytoskeleton, and/or on the interaction of granules and membrane with some

other, widely distributed, factor. Quantification also demonstrates that granule turnover by exocytosis is three times more marked in magnocellular nerve endings, which are therefore specialized for release by reason of their high surface membrane: volume geometry.

Release of neurosecretory dense-cored vesicles from the synaptic boutons of intrinsic hypothalamic neurones occurs preferentially at the membrane opposing the postsynaptic element, but about half of all such exocytosis occurs at unspecialized bouton membrane distant from the synaptic cleft; also from undilated axons.

By freezing exocytoses, tannic acid should prove a very valuable tool in the investigation of this ubiquitous, but elusive and poorly understood, event.

We thank the AFRC for financial support (AG 43/140), the Wellcome Trust for provision of an electron microscope, Lynne Ostle and Bob Wickens for skilful technical assistance, Drs D. W. Golding, F. D. Shaw, C. E. Lewis and A. Clark for helpful discussion, and the Company of Biologists for organizing *The Secretory Event*. Initial invertebrate work by DVP was supported by an SERC studentship.

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