PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Exocytosis and Membrane Recycling

J. Meldolesi and B. Ceccarelli

Phil. Trans. R. Soc. Lond. B 1981 **296**, 55-65
doi: 10.1098/rstb.1981.0171

Phil Trans. R. Soc. Lond. B **296**, 55–65 (1981)

Printed in Great Britain

Exocytosis and membrane recycling

By J. Meldolesi and B. Ceccarelli

Department of Pharmacology and C.N.R. Centre of Cytopharmacology, University of Milano, via Vanvitelli 32, *Milano, Italy* 20129

Exocytosis implies the fusion of the membrane of secretion granules with, and the insertion into, the plasmalemma. In non-growing systems such an insertion is temporary in that the inserted membrane is eventually removed.

Turnover results indicate that the removed membrane is not destroyed but recycled within the cell and reused. In some systems exocytosis occurs over the entire plasmalemma, while in others it is restricted to discrete regions, characterized by peculiar morphology and composition. Thus the fusion of the two membranes is probably preceded by a recognition step. Structural specializations were detected in interacting granule and plasma membranes by freeze–fracture and surface labelling techniques: arrays of intramembrane particles in protozoans and nerve terminals; clearing of particles and surface antigens in other systems. Direct evidence, obtained in some secretory systems, indicates that after exocytosis the granules and plasma membranes do not intermix, but remain segregated. The subsequent recapture of membrane patches of the granule type (in many systems by means of coated pits and vesicles) could then account for the striking specificity of the recycling process, documented by both composition and structural studies. In different systems the recycling of granule membranes is carried out at greatly different rates. Recent results in the parotid gland and neuromuscular junction indicate that this process is Ca^{2+}-dependent.

1. Introduction

It is now widely recognized that the mechanism whereby eukaryotic cells discharge their specific hydrophilic secretion products to the extracellular space is not molecular transport through the plasma membrane, but exocytosis. This process implies the fusion of the plasma membrane with the limiting membrane of specific cytoplasmic organelles containing secretion products in highly concentrated form. These organelles are designated as secretory granules in glandular and other secretory cells, and as synaptic vesicles in nerve endings of neurons. The fusion of the two independent membranes, which is restricted to a portion of their surface area, is followed by the destabilization of the fused membrane (fission). Thus, a continuity between the internal compartment of the organelle and the extracellular compartment is created and, consequently, the release of the organelle content is achieved (for reviews, see Palade (1975), Meldolesi *et al.* (1978), Orci & Perrelet (1978), Ceccarelli & Hurlbut (1980) and Orci (this symposium)).

For the overall economy of secretory cells, exocytosis is undoubtedly an extremely favourable process. Proteins and polypeptides (hormones, enzymes, etc.) are indeed able to cross membranes when they are in the form of extended chains, during their synthesis in the endoplasmic reticulum and before intracellular transport and storage within granules. However, they could never do so after acquiring their bulky three-dimensional final structure (Palade 1975; Blobel & Lingappa 1978; Scheele 1980). Smaller secretory products, such as acetylcholine

(ACh) and amine neurotransmitters, are able to cross membranes by simple diffusion along their electrochemical gradient. However, exocytosis might be as relevant in these systems as in glandular cells for it assures modulability (stimulation is coupled to transmitter release through exocytosis) and it provides the basis for a graded synchronous release of several multimolecular packets, or quanta, of neurotransmitter. These pulses escape from the axolemma at discrete sites, often opposite to strategically located regions on the postsynaptic membrane with a high concentration of specific receptor molecules (for reviews, see Heuser (1978), Ceccarelli & Hurlbut (1980*a*) and Winkler (1980)).

As already mentioned, exocytosis not only results in the discharge to the extracellular space of the granule (or vesicle) content, but also leads to the incorporation of the container – the limiting membrane – into the plasmalemma, with a consequent expansion of the cell surface. It was recognized early that in non-growing cell systems this expansion is at best a transient event, and it was therefore proposed that the centrifugal movement of membrane during exocytosis is balanced by a process of surface membrane removal. Beginning in the early 1970s the latter process (indicated hereafter as recycling) has attracted a great deal of research. The impressive body of biochemical and morphological evidence now available clearly indicates that recycling involves true centripetal (from the surface to the cytoplasm) movement and reutilization of the membrane taken up. In addition, it is now known that recycling serves to control not only the size but also the specificity of the cell surface, since it does not occur at random, but preserves the molecular identity of the participating membranes (for reviews, see Palade (1975), Meldolesi *et al.* (1978), Farquhar (1978), Heuser (1978), Ceccarelli & Hurlbut (1980*a*) and Herzog (1980 and this symposium)). This article is not intended to provide a complete account on exocytosis and recycling, for which the reader is referred to other recent comprehensive publications or reviews. We discuss findings that have become available during the last few years from our laboratories and which seem to us relevant to some open problems of the exo–endocytotic cycle.

2. Plasmalemma-granule membrane recognition: are specific 'receptors' involved?

At first glance, the question of specific recognition mechanisms between granule (or vesicle) and plasma membranes might appear to be merely a matter of common sense. In fact, the fusion of these two membrane types is highly specific in many respects: the nature of the membranes involved, and regulation by both stimulatory and inhibitory mechanisms. In addition, in many systems exocytosis does not occur over the entire cell surface but is restricted to specific areas (for example, the presynaptic membrane in neurons and the luminal region of the plasmalemma in exocrine cells). Thus the existence of a recognition step must necessarily be postulated. However, it should be acknowledged that our understanding of this process has not proceeded much beyond this elementary level. Quite a few years ago, the introduction of freeze–fracture in the study of secretion revealed in systems as different as protozoa and neuromuscular junctions the existence of peculiar intramembrane particle (i.m.p.) arrays at the sites of interaction between the plasmalemma and the membrane of secretory organelles, and opened the exciting possibility of a direct morphological identification of the specific recognition sites (Satir *et al.* 1973; Heuser *et al.* 1974. The subsequent refinements of these studies, however, failed to entirely fulfil these expectations. Thus, in Protozoa it became clear that i.m.p. arrays

are part of the complex structures that operate in docking secretory granules to the plasmalemma long before exocytosis takes place, and it is equally clear that they play an important role in stimulus–secretion coupling (Plattner *et al.* 1977, 1980; Matt *et al.* 1980). Furthermore, the establishment of such a complex arrangement requires not only the presence of the two interacting membranes, but also involves specific soluble proteins in the cytoplasm.

Analogously, at the frog neuromuscular junction it has been reported that, after electrical stimulation, fusion of synaptic vesicles is preferentially localized close to the regularly spaced structures of the presynaptic membranes, the active zones, which in freeze–fractured P faces appear as ridges running perpendicular to the major axis of the terminal, bordered on each side by a double row of large i.m.p. (Dreyer *et al.* 1973). Subsequent studies confirmed this preferential localization of vesicle fusion in terminals stimulated electrically or exposed to the major toxin of the black widow spider venom, α-latrotoxin (α-LT) (Ceccarelli *et al.* 1979*a*, *b*). It has also been reported that in terminals soaked for several hours in Ca^{2+}-free solutions the active zones become disorganized, and isolated remnants of the double rows of large i.m.p. can be found scattered about the presynaptic membrane. When α-LT was applied under these conditions, images of exocytosis were found both at relatively intact portions of the active zones and in the immediate vicinity of isolated remnants of the double rows (Ceccarelli *et al.* 1979*a*). However, when K^+ depolarization was used to stimulate exocytosis, a more complex picture emerged. Under this condition images of exocytosis were seen randomly distributed over the prejunctional membrane (Ceccarelli *et al.* 1978*b*). A similar random distribution has been reported for frog nerve terminals treated with brown widow spider venom in Ca^{2+}-free solutions (Pumplin & Reese 1977). Thus K^+ and brown widow spider venom seem to break the usual close association between the location of exocytotic sites and the double rows of particles. These two findings cast doubt on the predominant view that recognition sites are localized exclusively at the active zone. Active zones might play an important role in determining where exocytosis of transmitter occurs after some, but not all, stimuli to secretion. It is therefore possible that they represent restricted areas of the presynaptic membrane where access of vesicle to the axolemma is preferential, but not unique, at least under physiological conditions (see Ceccarelli & Hurlbut 1980*a*).

In other secretory systems, no morphological structures were observed for which a receptor function could be suggested. However, in the adrenal medulla, biochemical observations of at least potential interest have been reported. Thus, Mayer & Burger (1979) found that one protein, solubilized from a plasmalemma fraction by detergent treatment, binds aldehyde-fixed chromaffin granules immobilized onto Sepharose beads, and Mayer & Burger suggested that the protein might mediate the recognition step preceding fusion. An analogous function has been proposed by Pollard *et al.* (1979) for a soluble cytoplasmic protein, named synexin, also purified from the chromaffin tissue. At the moment these observations, although promising, appear too preliminary to allow conclusions as to the physiological role of these molecules.

3. Morphological identification of putative intermediate stages of membrane fusion–fission in exocytosis

Three successive stages of the membrane events leading to exocytosis were originally described by Palade (1975) on the basis of observations made on thin sections of epoxy-embedded secretory cells: close apposition (apposition) of the interacting membranes to yield a pentalaminar

structure (fusion); sequential elimination of the adjacent layers of the latter, with the final collapse of the hybrid membrane leading to the opening of the original site of fusion (fission). Actually, out of the intermediate stages, only apposition and fusion were commonly encountered in a variety of systems, and the others were therefore assumed to proceed spontaneously and very rapidly, thus escaping observation. The introduction of freeze–fracture led to an important development in the field. In fact, in several systems it was observed that after stimulation, granule and plasma membranes appeared completely devoid of i.m.p. over large surface areas of mutual apposition. At these sites cytochemical analyses revealed that some ectoproteins (such as lectin and Fc receptors) had been displaced from the plasmalemma (see Orci & Perrelet (1978) and Orci *et al.* (1981) for reviews). Moreover, in one system (B cells of the endocrine pancreas) exocytotic openings were convincingly observed within these i.m.p.-cleared areas (Orci *et al.* 1977). Taken together, these observations were interpreted to indicate that exocytosis is preceded by i.m.p. clearing of integral membrane proteins in both interacting membranes (a stage that might correspond to the pentalaminar organization seen in thin sections), and that therefore fission would develop between purely lipid bilayers (Orci *et al.* 1977, 1981; Orci & Perrelet 1978; Orci, this symposium).

However, a number of observations appear inconsistent with these conclusions. In fact, i.m.p. clearings were observed only in some secretory systems, and never found in others, such as the exocrine pancreas (Tanaka *et al.* 1980). In the frog neuromuscular junction, clearings were observed in peculiar experimental conditions, both at rest and during sustained secretion. These images might represent the close apposition of vesicles to the presynaptic membrane, but they do not seem to evolve toward exocytosis (Heuser 1976; Ceccarelli *et al.* 1979*a*). In other systems, clearings, but not openings in the cleared areas, were observed (see Tanaka 1980). Moreover, clearings develop not only between apposed granule and plasma membranes, but also between apposed membranes of two granules packed in the cytoplasm of unstimulated goblet, parotid or lacrimal cells (Neutra & Schaeffer 1977; Tanaka *et al.* 1980) or even brought together *in vitro* by centrifugation (Schuler *et al.* 1978; Tanaka *et al.* 1980). In the latter condition the frequency of i.m.p.-cleared areas in parotid granule membranes was found to vary depending on the centrifugal force applied (Tanaka *et al.* 1980). Finally, results obtained in unfixed cells by using the technique of quick freezing cast doubt even on the reality of i.m.p. clearings, in that early openings revealed by this technique were found to be much smaller than in fixed specimens and surrounded by i.m.p.-studded membranes. These differences between fixed and unfixed specimens led Chandler & Heuser (1979, 1980) to conclude that i.m.p. clearing might be an artefact introduced by aldehyde fixation. At present, a definite interpretation of all these apparently conflicting results is not easy. In fact, on the one hand, it seems plausible that aldehyde fixation does indeed interfere with the morphology of actively secreting cells. For example, it has been reported that, at the frog neuromuscular junction, fusion of synaptic vesicles continues for some time while fixation is occurring and that vesicles discharged during this time are trapped fused with the plasmalemma. Thus, the number of fusions appearing in fixed specimens can be envisaged as an integral of the events occurring during the fixation time (Ceccarelli *et al.* 1979*a*; Ceccarelli & Hurlbut 1980*a*). On the other hand, images of i.m.p. clearing seem to be real, at least in some instances, for such images have been observed in between apposed granules of both goblet and parotid cells without fixation (Specian & Neutra 1980; Tanaka *et al.* 1980). The available evidence therefore suggests that i.m.p. clearing may result from the simple apposition between two membranes and that in some systems

granule–plasmalemma apposition precedes exocytosis, possibly not as an irreversible intermediate, but only as a preliminary and reversible step. Aldehyde fixation could favour and maintain such membrane apposition and possibly extend the surface area of cleared membranes.

In conclusion, even if interesting findings have been recently accumulated, the interpretation of the morphological results on membrane interactions during exocytosis is not yet settled, and many important aspects of the process remain totally obscure.

4. Membrane recycling: kinetics and mechanisms

So far, very few studies have dealt with the direct investigation of the kinetics of the membrane events attending either exocytosis or recycling. In fact, much of the available information on these processes comes from morphological studies that were often analysed in non-quantitative terms. For exocytosis, however, an indirect approach has been widely used, i.e. the monitoring of discharged secretory products, carried out either by biochemical assay or by the estimation of the elicited physiological responses. Clearly, results obtained by this approach can be straightforward only in secretory systems where exocytosis is the only process by which the secretory product is discharged. If other processes coexist, such as the direct diffusion through the plasmalemma (see § 1), these studies are much more complex, but often the results are still suitable for quantitative analysis. Similar, but even greater, problems arise with recycling, because retrieval of membranes from the cell surface to the cytoplasm does not occur exclusively as a consequence of exocytosis. In fact it is now well recognized that other types of endocytosis, such as fluid-phase and receptor-mediated endocytosis, do exist in eukaryotic cells, including those specialized towards secretion. Thus, the simple quantitative measurement of a tracer taken up by secretory cells, even in the course of specified stages of their secretory activity, cannot provide separate information on the kinetics of individual forms of endocytosis. Studies aimed to investigate the kinetics of post-exocytotic membrane recycling necessarily imply the quantitative evaluation of the amount of membrane in the various subcellular compartments participating in the process, particularly in the plasmalemma.

An important difference among various secretory systems emerges from the simple comparison of the morphology of stimulated and unstimulated cells. In fact, in some systems the surface area of the plasmalemma (or of the region to which exocytosis is restricted) is not greatly modified even during maximal stimulation; on the other hand, in other systems this area is considerably increased. This observation suggests that in the former systems (exocrine and endocrine pancreatic cells, somatotroph and mammotroph cells of the pituitary gland, etc.) exocytosis is matched by recycling, whereas in the latter systems (acinar cells of the parotid and lacrimal glands, mast cells, goblet cells) the balance between exocytosis and recycling can be broken, with a consequent temporary accumulation of granular membranes at the cell surface. We have recently taken advantage of such a partial dissociation between exocytosis and recycling to investigate the time course of membrane removal from the plasmalemma after a period of sustained stimulation *in vitro*. Tissue lobules of parotid gland were first stimulated by a β-adrenergic agonist until most of the prestored granules had been discharged, then lobules were incubated in the presence of a β-blocker (to inhibit residual exocytosis) and the decrease with time of the luminal plasmalemma area was monitored in thin sections. Our data indicate that retrieval of membrane from the plasmalemma follows apparent first-order kinetics, with

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

a half time of about 18.5 min. Whether similar kinetics of membrane removal is followed by parotid acinar cells also at rest or during different rates of secretion is totally unknown (Koike & Meldolesi 1981).

A suitable system for a detailed analysis of the processes of exocytosis and recycling is the frog neuromuscular junction, since secretion can be graded with good accuracy by varying the rate of electrical nerve stimulation, and its time course can be continuously followed by recording intracellularly the postsynaptic electrical activity. Two important features of recycling were revealed by studies carried out in this system. At a low frequency of stimulation (2 Hz, which corresponds to an initial rate of release of approximately 400 ACh quanta per second) the terminals could be stimulated for several hours without suffering any appreciable signs of membrane redistribution from the internal to the axolemma compartment (net loss of synaptic vesicles, swelling and formation of deep infoldings of the axolemma). Thus, under these conditions of stimulation, exocytosis and recycling were in balance and the average time spent by fused vesicles in the axolemma could not be longer than a few seconds. However, if the frequency of stimulation used was increased to 10 Hz, the picture that emerged was totally different: quantal release of ACh subsided progressively, to be virtually abolished within 20 min. At this time terminals were swollen, infoldings of axolemma had developed and nerve terminals were profoundly depleted of synaptic vesicles. These changes in structure were reversed after a rest of several minutes (Ceccarelli *et al.* 1973; Ceccarelli & Hurlbut 1980*a*).

Taken together, these results indicate that the mechanism of recycling operating at a low frequency of stimulation is overwhelmed at a high frequency, and that accumulation of membrane into the axolemma during stimulation at 10 Hz occurred because membrane was added faster than it could be removed. The depletion observed under these conditions may be the result of the reduced speed in the recovery process, with a consequent increase in the average time spent by the vesicle membranes in the axolemma. At the moment it is impossible to exclude the possibility that this delay is simply due to a partial impairment of the fast recycling process operating at low frequency. However, the intriguing possibility does exist that, under the two conditions of stimulation mentioned above, recycling is maintained by two different processes (see Ceccarelli & Hurlbut 1980*a*). In this respect it is interesting that experimental evidence supporting the existence of two mechanisms of membrane recycling has been reported at the frog neuromuscular junction. The first of these mechanisms would consist of the quick direct removal of vesicle membranes immediately after fusion, without intervening flattening into the axolemma, whereas by a second different mechanism vesicles would first become completely incorporated into the axolemma and then recycled as coated vesicles. That the first of these mechanisms might account for the fast recycling occurring at low frequency, and that the second might predominantly operate at high frequency of stimulation, is suggested by the paucity of coated vesicles described in the first condition (Ceccarelli *et al.* 1973; Ceccarelli & Hurlbut 1975) and by the increase that has been reported in the second condition (Heuser & Reese 1973; Heuser 1976, 1978). This suggestion is strengthened by the simple consideration that the process elicited by high-frequency stimulation appears to operate at rates of the same order of magnitude as those operating in glands, such as the parotid gland (see above), as well as in receptor-mediated endocytosis; in these two conditions coated vesicles are known to be involved (Herzog & Farquhar 1977; Herzog 1980; Willingham *et al.* 1981; Salsbury *et al.* 1980).

One emerging question is why two separate recycling mechanisms should be needed at the neuromuscular junction, and possibly also in other types of nerve terminals. As already

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

mentioned in the introduction, recycling is a complex process by which the input of membrane to the plasmalemma is balanced not only in quantity but also in quality (see Palade 1975; Meldolesi *et al.* 1978). Thus, the existence of specific sorting-out mechanisms able to discriminate and remove patches (or molecular components) of the two participating membranes must be postulated to prevent their random intermixing, otherwise expected on the basis of the current models of membrane structure. Although in some secretory systems the two participating membranes maintain their morphological identity, at least for some time after fusion (De Camilli *et al.* 1976; Chandler & Heuser 1979, 1980), direct experimental information on the phenomena occurring in the plane of the membranes is very limited. Indeed it appears that if intermixing does occur it might be incomplete, proceed slowly and possibly involve only some of the molecular components of the two fused membranes.

These considerations might be useful in understanding the different results reported at frog neuromuscular junctions. In this system the secretory organelles – the synaptic vesicles – are small, of about the same size as the recycled vesicles, and they contain a highly diffusible secretory product. Even transient fusion with the axolemma could therefore allow transmitter escape and concomitantly recover a population of vesicles, available for subsequent release, with little or no chance of intermixing of membrane components. We have postulated that this mechanism operates at low frequencies of stimulation. In addition it should be emphasized that this direct recycling would undoubtedly be thermodynamically favourable, since it would save the work required for the invagination of patches of flattened membrane (see Ceccarelli & Hurlbut 1980*a*) (figure 1*a*).

If, however, the direct recycling system were to become inefficient (as might occur at high frequency of stimulation), more and more synaptic vesicles would collapse into the axolemma (figure 1*b*), thus leading to at least two consequences: the loss of the geometry favourable to direct recycling and, possibly, the occurrence of some membrane intermixing. From these two points of view, the situation at the neuromuscular junction would become analogous to that of glandular cells, which discharge large granules and recycle small vesicles relatively slowly (figure 1*c*). It is therefore not surprising that recycling might involve coated vesicles in both cases. In fact, coated vesicles are recognized to operate as membrane molecular filters, i.e. to originate by pinching off from membrane domains where specific components are concentrated, while others are excluded (Bretscher *et al.* 1980). In glands the preferential origin of coated vesicles from plasmalemma patches corresponding to previously discharged granules has been recognized (Koike *et al.* 1980) (figure 1*c*). Thus, coated vesicles might constitute a refined control mechanism that guarantees the fidelity of the granule membrane to be recycled and reused in subsequent secretory cycles.

If recycling does indeed occur as hypothesized above, one needs to postulate that in each secretory system the membrane of coated vesicles is identical to that of the secretory granule or vesicle. This possibility has been questioned on the basis of results obtained in cultured fibroblasts, where large coated vesicles (involved in receptor-mediated endocytosis of lipoproteins) exhibit in freeze–fracture a high density of large P face i.m.p. (Orci *et al.* 1978, 1981). The structure of these vesicle membranes appears quite different from that of secretory granules membranes, which is usually very poor in i.m.p. on both fracture faces. However, fibroblasts are not typical secretory cells, and it is therefore possible that most of their coated vesicles (and in particular those of large size participating in receptor-mediated endocytosis) are not involved in the post-exocytotic membrane recycling. In contrast, at the luminal plasmalemma of mouse

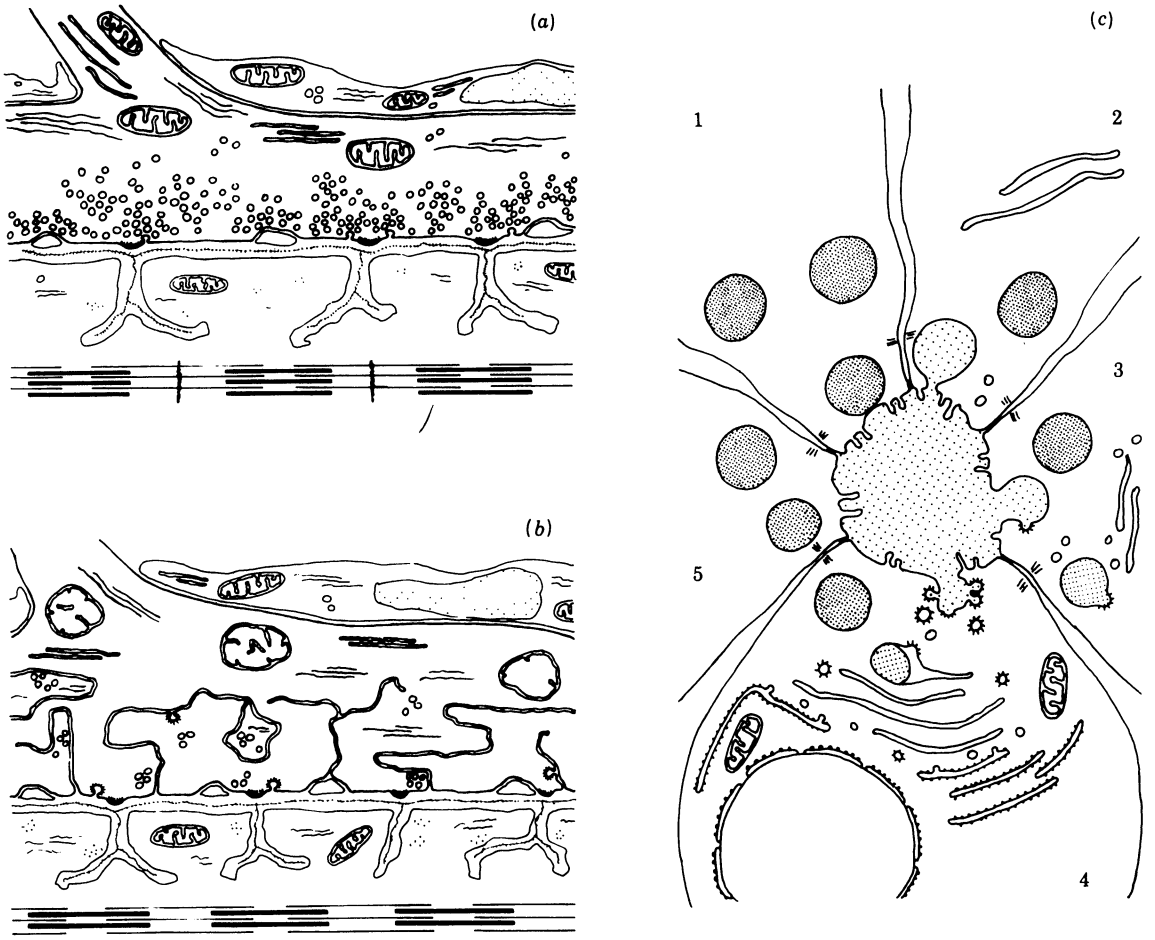

FIGURE 1. Schematic representation of exocytosis and endocytosis at the frog neuromuscular junction stimulated electrically at two frequencies: 2 Hz (*a*) and 10 Hz (*b*) as well as in an exocrine gland (*c*). In terminals stimulated up to 4 h at low frequency (*a*) the general ultrastructure is similar to that of resting terminals. Synaptic vesicles are numerous, the size of the ending is normal and the axolemma does not exhibit membrane infoldings. Continuities between vesicles and plasma membrane are visible only at the active zones. These findings are interpreted to mean that in those conditions exocytosis and recycling are in balance, because each discharging synaptic vesicle is rapidly (at the most within seconds) recycled to the intraterminal compartment, without intervening flattening in the presynaptic membrane. Such a direct recycling is expected to prevent the chances of molecular intermixing of vesicle and presynaptic membranes. The appearance of terminals stimulated at high frequency (*b*) is different. Within 2 min of stimulation, large quantities of membrane appear to have been redistributed from synaptic vesicles (which are greatly diminished in number) to the axolemma (which is swollen and shows numerous profound infoldings, resulting in a quasi-complete septation of the terminal). Numerous coated vesicles have been described under these conditions. Some of them were seen budding from the presynaptic and the infolding membrane. These changes were reversed by resting the preparation for several minutes. It therefore appears that under high-frequency stimulation the balance between exocytosis and endocytosis is broken, so that fused synaptic vesicles flatten in the presynaptic membrane and remain incorporated into the surface membrane compartment for prolonged periods of time (minutes). At this point recycling might require the involvement of coated pits, acting as molecular filters, to preserve the molecular fidelity of the vesicle to be retrieved. Such a situation appears similar to that of glandular cells, which discharge large organelles (secretion granules) and recycle small coated vesicles as depicted in (*c*) for exocrine cells. In cell 1 a secretion granule approaches the luminal surface of the plasmalemma and then fuses over a limited portion of its surface (exocytosis, cell 2). This stage is followed by a transformation of the exocytosis from the flask to the omega shape, with the concomitant appearance of forming coated pits over part of its surface (3). Eventually, clusters of coated pits are seen originating from the membrane of the discharged granule (4), so that finally the membrane is completely retrieved and the situation preceding exocytosis is re-established (5). Studied in parotid acinar cells stimulated *in vitro* with the β-adrenergic agonist isoprenalin, the retrieval of granule membrane was found to proceed according to first-order kinetics, with an approximate half time of 18.5 min (Koike & Meldolesi 1981).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

pancreatic acinar cells, where clusters of coated pits originate from membrane infoldings still recognizable as discharged zymogen granules, the limiting membrane was found to be as poor in i.m.p. as that of granules (Koike *et al.* 1980). These observations emphasize the possibility that coated vesicles, although surrounded by similar coats, might in fact be heterogeneous as to their membrane, since they are not involved in one single function but in a variety of functions (examples are various types of endocytosis, transport of secretory products from the endoplasmic reticulum to Golgi elements and condensing vacuoles (see Farquhar 1978; Herzog 1980, Salsbury *et al.* 1980; Willingham *et al.* 1981).

5. Recycling as an independently regulated process

The emergence of recycling as a process coupled to, and yet to some extent independent from, exocytosis, raises the question of its regulation. The information as yet available is, however, very limited, mostly because the strict connection of recycling to exocytosis represents a formidable obstacle to the development of adequate experimental protocols. In addition, it appears possible that the regulation of recycling is not exactly the same in all secretory systems. For example, it has been reported by Herzog & Reggio (1980) that horseradish peroxidase, infused into the pancreatic duct system under conditions where acinar lumina are reached, stops the formation of coated vesicles and induces the appearance of a type of endocytosis by small vesicles that move intracellularly to the lysosomal, rather than to the Golgi, compartment. In contrast, at the frog neuromuscular junction horseradish peroxidase has apparently no effect on synaptic vesicle recycling and reutilization (Ceccarelli *et al.* 1973).

Recently, the two systems described in the above sections were used in our department to investigate the Ca^{2+} dependence of recycling. The original observation was made by Ceccarelli & Hurlbut (1980*b*) working on the frog neuromuscular junction exposed to α-LT at doses even as low as 0.4 μg/ml. This toxin has the peculiar power to stimulate a massive asynchronous discharge of ACh quanta from terminals incubated in Ca^{2+}-free solution. After 45–60 min of exposure to α-LT in Ca^{2+}-free solution, the quantal secretion is exhausted and the morphological analysis reveals a nearly complete disappearance of synaptic vesicles, coupled with a marked swelling of the terminals. These findings were interpreted to mean that α-LT might have a dual effect: not only a stimulation of transmitter release but also a blocking of vesicle recycling. However, when α-LT was applied in normal Ringer solution, a completely different picture emerged: although the initial rate of release was apparently as high as in the Ca^{2+}-free solution, it did not become exhausted during an hour nor was the general morphology of the terminal appreciably modified with respect to the resting state. Moreover, if horseradish peroxidase was added to the incubation fluid, most vesicles were labelled with the tracer, indicating that they had undergone cycles of exo–endocytosis throughout the experiment (Ceccarelli & Hurlbut 1980). It appears therefore that the presence of Ca^{2+} in the extracellular fluid is essential for maintaining recycling, i.e. that recycling is Ca^{2+} dependent. It is possible that at the frog neuromuscular junction the requirement of Ca^{2+} for endocytosis is general and not restricted to α-LT-induced quantal secretion. Thus, a rapid depletion of vesicles might occur whenever secretion is stimulated in Ca^{2+}-free solutions.

Further support to the generality of Ca^{2+} requirement in recycling comes from results subsequently obtained in the parotid gland. These experiments were carried out according to the protocol described above (see the second paragraph of §4), but Ca^{2+}-free Ringer solution (with 1 mM EGTA) was used as the medium during the post-stimulatory incubation. Under

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

these conditions the rate of membrane retrieval from the luminal surface of acinar cells was considerably slower (less than one quarter) than that observed in the controls. Interestingly, when lobules were shifted from the Ca^{2+}-free to the complete Ringer medium, this effect was readily reversed, in the sense that the rate of recycling returned approximately to its control value (Koike & Meldolesi 1981).

6. Conclusions

This review has emphasized the importance of morphological studies, particularly fruitful when coupled with biochemical and biophysical investigations. The results obtained by such an approach during the last few years have greatly broadened our knowledge on both exocytosis and, especially, recycling, to the point that a general framework can now be proposed where established facts are linked together by reasonable hypotheses. However, necessary conditions for a widespread acceptance of these hypotheses are still lacking. Two problematic aspects of the studies on exocytosis and recycling are worth mentioning here. On the one hand, the highly integrated nature of the processes at hand, which occur only in intact cellular preparations, has precluded so far any adequate reproduction, or experimental dissection, in cell-free conditions. On the other hand, there is a need for the introduction of new research technology and for the refinement and widespread application of techniques already available, such as quick freezing and immunocytochemistry at the electron microscopical level, which so far have been exploited to a limited extent with respect to their large potential.

We wish to express our appreciation to the following colleagues, who participated in the experimental work summarized in this article: P. De Camilli, R. Fesce, F. Grohovaz, W. P. Hurlbut, H. Koike and Y. Tanaka. Special thanks are due to Dr R. Fesce for drawing the figure.

References (Meldolesi & Ceccarelli)

Blobel, G. & Lingappa, V. 1978 Transfer of protein across intracellular membranes. In *Transport of macromolecules in cellular systems* (ed. S. C. Silverstein), pp. 289–298. Berlin: Dahlem Konferenzen.

Bretscher, M. S., Thomson, J. N. & Pierce, B. M. F. 1980 Coated pits act as molecular filters. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4156–4159.

Ceccarelli, B., Grohovaz, F. & Hurlbut, W. P. 1979*a* Freeze–fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. I. Effects of black widow spider venom and Ca^{2+}-free solutions on the structure of the active zone. *J. Cell Biol.* **81**, 163–177.

Ceccarelli, B., Grohovaz, F. & Hurlbut, W. P. 1979*b* Freeze–fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. II. Effects of electrical stimulation and high potassium. *J. Cell Biol.* **81**, 178–192.

Ceccarelli, B. & Hurlbut, W. P. 1975 The effect of prolonged repetitive stimulation in hemicholinium on the frog neuromuscular junction. *J. Physiol., Lond.* **247**, 163–188.

Ceccarelli, B. & Hurlbut, W. P. 1980*a* Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* **60**, 396–441.

Ceccarelli, B. & Hurlbut, W. P. 1980*b* Ca^{2+} dependent recycling of synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* **87**, 297–303.

Ceccarelli, B., Hurlbut, W. P. & Mauro, A. 1973 Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* **57**, 499–524.

Chandler, D. E. & Heuser, J. E. 1979 Membrane fusion during secretion. Cortical granule exocytosis in sea urchin eggs as studied by quick freezing and freeze–fracture. *J. Cell Biol.* **83**, 91–108.

Chandler, D. E. & Heuser, J. E. 1980 Arrest of membrane fusion events in mast cells by quick freezing. *J. Cell Biol.* **83**, 666–674.

De Camilli, P., Peluchetti, D. & Meldolesi, J. 1976 Dynamic changes of the lumenal plasmalemma in stimulated parotid acinar cells. *J. Cell Biol.* **70**, 59–74.

Dreyer, F., Paper, K., Akert, K., Sandri, C. & Moor, H. 1973 Ultrastructure of the 'active zone' in the frog neuromuscular junction. *Brain Res.* **62**, 373–380.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Farquhar, M. G. 1978 Traffic of products and membranes through the Golgi complex. In *Transport of macromolecules in cellular systems* (ed. S. C. Silverstein), pp. 341–362. Berlin: Dahlem Konferenzen.

Herzog, V. 1980 Role of the Golgi complex in intracellular transport. In *Biological chemistry of organelle formation* (ed. T. Bücher, W. Sebald & H. Weiss), pp. 119–145. Berlin: Springer-Verlag.

Herzog, V. & Farquhar, M. G. 1977 Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5073–5077.

Herzog, V. & Reggio, H. 1980 Pathways of endocytosis from luminal plasma membrane in rat exocrine pancreas. *Eur. J. Cell Biol.* **21**, 141–180.

Heuser, J. E. 1976 Morphology of synaptic vesicle discharge and reformation at the frog neuromuscular junction. In *Motor innervation of muscle* (ed. S. Thesleff), pp. 51–115. London: Academic Press.

Heuser, J. E. 1978 Synaptic vesicle exocytosis and recycling during transmitter discharge from the neuromuscular junction. In *Transport of macromolecules in cellular systems* (ed. S. C. Silverstein), pp. 445–464. Berlin: Dahlem Konferenzen.

Heuser, J. E. & Reese, T. S. 1973 Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **57**, 315–344.

Heuser, J. E., Reese, T. S. & Landis, D. M. D. 1974 Functional changes in frog neuromuscular junctions studied with freeze–fracture. *J. Neurocytol.* **3**, 109–131.

Koike, H. & Meldolesi, J. 1981 Post-stimulation retrieval of luminal surface membrane in parotid acinar cells is calcium-dependent. *Expl Cell Res.* **134**, 375–386.

Koike, H., Tanaka, Y., Réz, G. & Meldolesi, J. 1980 Membrane interactions in pancreatic acinar cells: exocytosis, recycling and autophagocytosis. In *Biology of normal and cancerous pancreatic cells* (ed. A. Ribet, L. Pradayrol & C. Susini), pp. 215–228. Amsterdam: Elsevier/North-Holland.

Matt, H., Plattner, H., Reichel, K., Lefort-Tran, M. & Beisson, J. 1980 Genetic dissection of the final exocytosis steps in *Paramecium tetraurelia* cells: trigger analysis. *J. Cell Sci.* **46**, 41–60.

Mayer, D. I. & Burger, M. M. 1979 Isolation of a protein from the plasma membrane of adrenal medulla which binds to secretory vesicles. *J. biol. Chem.* **254**, 9854–9859.

Meldolesi, J., Borgese, N., De Camilli, P. & Ceccarelli, B. 1978 Cytoplasmic membranes and the secretory process. In *Membrane fusion* (ed. G. Poste & G. N. Nicolson), pp. 510–627. Amsterdam: North-Holland.

Neutra, M. R. & Schaeffer, S. F. 1977 Membrane interactions between adjacent mucous secretion granules. *J. Cell Biol.* **74**, 983–991.

Orci, L. & Perrelet, A. 1978 Ultrastructural aspects of exocytotic membrane fusion. In *Membrane fusion* (ed. G. Poste & G. N. Nicolson), pp. 630–657. Amsterdam: North-Holland.

Orci, L, Carpentier, J. L., Perrelet, A., Anderson, R. W. G., Goldstein, J. L. & Brown, M. L. 1978 Occurrence of low density lipoprotein receptors within large pits on the surface of human fibroblasts as demonstrated by freeze–fracture. *Expl Cell Res.* **113**, 1–13.

Orci, L., Perrelet, A. & Friend, D. S. 1977 Freeze–fracture of membrane fusions during exocytosis in pancreatic B cells. *J. Cell Biol.* **75**, 23–30.

Orci, L., Montesano, R. & Perrelet, A. 1981 Exocytosis–endocytosis. In *Basic mechanisms of cellular secretion* (ed. A. R. Hand & C. Oliver). New York: Academic Press. (In the press.)

Palade, G. E. 1975 Ultrastructural aspects of the process of protein secretion. *Science, N.Y.* **189**, 347–358.

Plattner, H., Reichel, K., Matt, H., Beisson, J., Lefort-Tran, M. & Pouphile, M. 1980 Genetic dissection of the final exocytosis step in *Paramecium tetraurelia* cells. Cytochemical determination of Ca^{2+} ATPase activity over preformed exocytosis. *J. Cell Sci.* **46**, 17–40.

Pollard, H. B., Pazoles, C. J., Creutz, C. E. & Zinder, O. 1979 The chromaffine granule and possible mechanisms of exocytosis. *Int. Rev. Cytol.* **58**, 160–197.

Pumplin, D. W. & Reese, T. S. 1977 Action of brown widow spider venom and botulinum toxin on the frog neuromuscular junction examined by freeze–fracture technique. *J. Physiol., Lond.* **273**, 443–457.

Salsbury, J. L., Condeelis, J. S., Satir, P. 1980 The role of coated vesicles, microfilaments and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. *J. Cell Biol.* **87**, 132–144.

Scheele, G. 1980 Biosynthesis, segregation and secretion of exportable proteins by the exocrine pancreas. *Am. J. Physiol.* **238**, G467–477.

Schuler, G., Plattner, H., Aberer, W. & Winkler, H. 1978 Particle segregation in chromaffin granule membranes by forced physical contact. *Biochim. biophys. Acta* **512**, 244–254.

Specian, R. D. & Neutra, M. R. 1980 Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. *J. Cell Biol.* **85**, 626–640.

Tanaka, Y., De Camilli, P. & Meldolesi, J. 1980 Membrane interactions between secretion granules and plasmalemma in three exocrine glands. *J. Cell Biol.* **84**, 438–453.

Willingham, M. C., Haigler, H. T., Dickson, R. B. & Pastan, I. H. 1981 Receptor-mediated endocytosis in cultured cells: coated pits, receptosomes and lysosomes. In *International cell biology 1980–1981* (ed. H. G. Schweiger), pp. 613–621. Berlin: Springer-Verlag.

Winkler, H. & Westhead, E. 1980 The molecular organization of adrenal chromaffin granules. *Neuroscience* **5**, 1803–1823.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES