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COMMENTARY

INTRACELLULAR MECHANISMS IN EXOCYTOTIC SECRETION

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Understanding the exocytotic process is of profound importance for understanding a spectrum of phenomena, ranging from unicellular defense mechanisms to endocrine regulatory functions, neurotransmission, and killer cell action. Elucidation of the cellular mechanisms constituting the process of secretion has been thwarted, however, by their protection inside the intact cell. The complexity of the process has hindered its reconstitution *in vitro*. Thus, permeabilization of the plasma membrane with preservation of secretion has led to enthusiastic activity in the investigation of stimulated secretion.

Chromaffin cells, best known for their secretion of adrenaline under situations of stress, have been used to study stimulated secretion of hormones *in vivo*, in the perfused gland, in tissue sections, and in primary cell cultures. The possibility of obtaining large amounts of homogeneous tissue has enabled the purification of several proteins putatively involved in secretion. The secretory process in chromaffin cells has several characteristics in common with secretion in other cells, but it is becoming increasingly clear that exocytosis is regulated differently in various cell types. One of the main differences seems to exist between the secretory mechanisms of excitable versus non-excitable cells. In mast cells for example, Ca^{2+}, the common second messenger of excitable cells, has been proven to be neither sufficient nor necessary for secretion. In this commentary we will discuss recent data that suggest the excitable chromaffin cell as an excellent model system for the study of fast excitation–secretion coupling. The combination of biochemical and electrophysiological techniques in this system may provide new clues to the understanding of neurotransmitter secretion.

The calcium dependence of secretion

The application of cell membrane permeabilization techniques in the field of chromaffin cell biology has led to interesting findings [1–3]. Bypassing the signal pathway from receptor occupation, membrane depolarization and Ca^{2+} influx, down to the introduction of Ca^{2+} through artifical pores apparently does not affect the final secretory output. Equal amounts of catecholamines can be secreted by permeabilized cells upon raising the free Ca^{2+} to micromolar concentrations and by intact cells upon stimulation with the physiologically relevant transmitter acetylcholine. The secretory responses in both cases show similar kinetics, with half-maximal values reached within minutes of stimulation. This has been interpreted uncritically as activation of the same intracellular mechanisms. Secretion in permeabilized cells shows surprisingly little specificity for ions and nucleotides. In essence, Mg^{2+}-ATP proves to be sufficient when it is induced by elevation of the intracellular free Ca^{2+} concentration. Individual Ca^{2+}- or Mg^{2+}-ATP-dependent steps, however, could not be characterized.

Since cells permeabilized by detergents or bacterial toxins lose the bulk of soluble cytoplasmic components [2–4], the secretory machinery must be amazingly stable. Partial responses to raising the Ca^{2+} concentration can be measured for periods up to 1 hr following permeabilization [5]. This indicates that either a portion of the chromaffin granules, the secretory vesicles of chromaffin cells, remains in a stable, fusion competent configuration and/or the key elements of the secretory process diffuse from their proper site of action only slowly.

The steps of secretion

The secretory process can be subdivided into three phases. The *initial phase* comprises synthesis, modification and sorting of the molecules to be secreted. In a *transport phase*, these secretory products, packaged into vesicles, are translocated to the cell periphery by mechanisms not yet identified. The *peripheral phase* includes two opposing principles, namely inhibition versus promotion of fusion between the secretory vesicles and the plasma membrane. It is the inhibitory mechanism which guarantees availability of vesicles at the fusion site and thus represents the key element in stimulated secretion. The other principle promotes the fusion of the two opposed membrane bilayers which may well be based on mechanisms common to sundry membrane fusion processes.

Reorganization of the peripheral filament network. The peripheral phase can be viewed as a succession of individual steps (as displayed in Fig. 1) representing levels of control for fine-tuning of the system. The first inhibitory elements encountered by the chromaffin granule on its translocation towards the eventual fusion sites seem to be the subplasmalemmal cytoskeletal network [6]. This dense network, primarily composed of actin filaments, may

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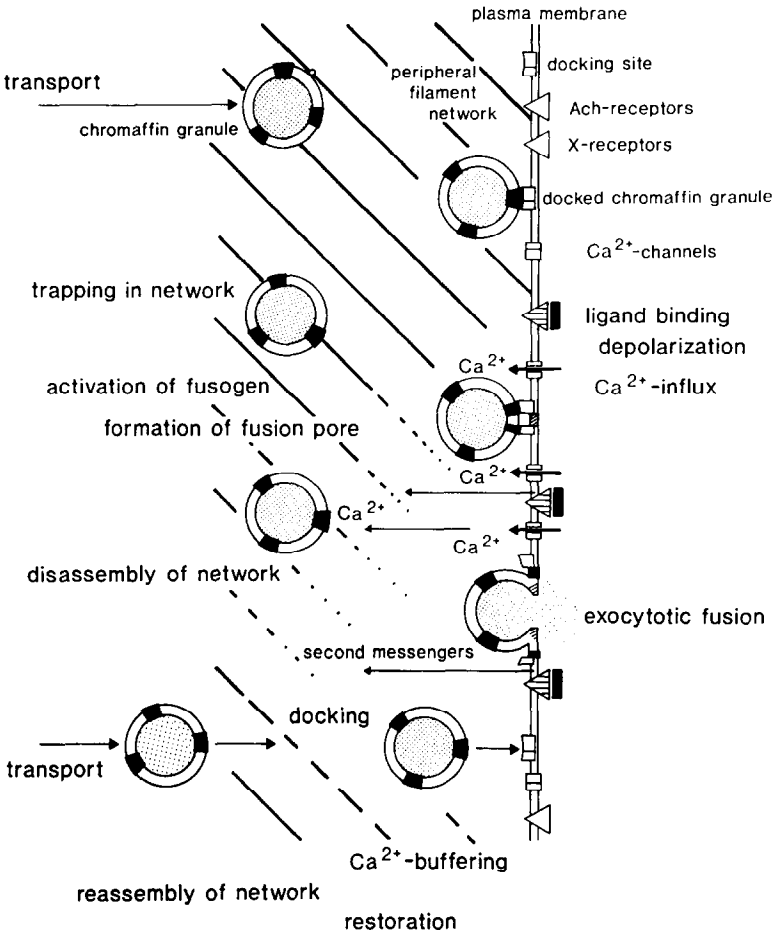

Fig. 1. Scheme of steps involved in the peripheral phase of stimulated secretion. Chromaffin granules transported to the cell periphery are hindered by a microfilament network from proceeding to the plasma membrane. Only upon rearrangement of this network can granules advance to the cell membrane and dock in a stable configuration. Transmembrane signalling produces intracellular second messengers which induce exocytotic fusion of chromaffin granule and plasma membrane. A detailed description and discussion of the steps is given in the text.

directly hinder the chromaffin granules from advancing to the plasma membrane. Specific binding to this cytoskeleton through membrane-located actin-binding proteins could restrain chromaffin granules from further translocation and diffusion, thus accumulating them in the cell periphery. Only after local disassembly of the network can granules proceed close to the cell membrane. A general, transient disassembly of the peripheral actin network in response to severe stimulation has been observed [7]. Disintegration of the network can be prompted by raising intracellular cAMP levels. It is independent of intracellular Ca^{2+} concentrations and is not sufficient to induce secretion. Only in conjunction with a rise in Ca^{2+} does disassembly of the network result in maximal secretory activity [8].

The link between granule membrane and the filament network is postulated to be accomplished by the actin-binding protein α-fodrin, localized in the chromaffin cell periphery [9]. Functional involvement in exocytosis is inferred from experiments in which the introduction of anti-α-fodrin antibodies into detergent-permeabilized chromaffin cells inhibited secretion of catecholamines by about 50% [10]. The role of α-fodrin could not be defined more precisely, and the effect of antibodies on the actin network under these conditions was not investigated. The partial block of secretion by anti-α-fodrin antibodies together with the effects of various stimuli on disassembly and/or secretion are consistent with a gating function of the actin filament network in the cell periphery. The regulation of disassembly and reassembly however, remains to be established.

Docking of chromaffin granules. Local reorganization of the dense peripheral network, possibly together with directional transport and docking of the granules to the plasma membrane prior to fusion, may restrict secretion to specific regions of the plasma membrane. Such localized secretion, a characteristic of synaptic nerve endings, was observed recently in chromaffin cells cocultured with fibroblasts [11]. Subpopulations of vesicles in close contact, or docked to the plasma membrane, are displayed in a variety of systems such as the neuro-

muscular junction [12], the synapses between parallel fibers and Purkinje cells [13], and the unicellular organism *Paramecium* [14]. In PC 12 cells, a rat pheochromocytoma cell line, which contain a high proportion of docked vesicles, the can clearly be recognized lined up along the plasma membrane in electron micrographs, even when cells are permeabilized [15]. Morphological evidence for docked granules also has been obtained recently in chromaffin cells [16]. The docking complex may be composed of receptor–ligand pairs specific for the chromaffin granules and the plasma membrane [17]. A putative component o the docking complex has been isolated from chromaffin cell plasma membranes [18]. Functional involvement of this protein in a step distal to Ca^{2+}-influx was shown in single cells [19] (see below).

Docking could have multiple functions in stimulated secretion. First, it can contribute to spatial specificity of secretion. Second, it can represent the final inhibitory mechanism to block spontaneous exocytotic fusion of the two membranes. Third, docking of granules can allow an immediate reaction to stimulation. The docked, supposedly fusion competent granule is held in close contact with the plasma membrane ready to react to an appropriate signal like an arrow in a bent bow. Locking a responsive system in a final step allows it to meet all time- and energy-consuming requirements long before triggering. This enables the cell to respond instantaneously to a stimulus by secretion of part of the stored catecholamines.

Activation of the fusion process. A possible interference from docking is that both interacting membranes are "primed" for fusion, and that stimulation of the cell releases a block between them to initiate exocytosis. Physiological stimulation leads to membrane depolarization and Ca^{2+} influx. Depolarization in the absence of Ca^{2+} influx has been shown to contribute to exocytotic fusion in a neuromuscular junction of a crayfish [20]. In chromaffin cells secretion crucially depends on the influx of Ca^{2+}. Interestingly, the introduction of Ca^{2+} through ionophores in intact cells does not lead to the full secretory response [21], whereas the introduction of high amounts of Ca^{2+} through a patch pipette induces exocytosis even in the absence of depolarization [22]. In a cell-free system, isolated chromaffin granules and plasma membrane fractions were shown to aggregate, but fusion could not be induced by raising the Ca^{2+} concentration [23]. This suggests that either correct docking was not attained *in vitro*, the two membranes were not fusion competent, or the block between the membranes could not be removed by Ca^{2+} alone.

Alternatively, the plasma membrane may not be fusion competent, but fusogenic components in this membrane may be generated only upon stimulation of the cell. In permeabilized PC 12 and chromaffin cells, the increase in Ca^{2+} to micromolar concentrations apparently is sufficient to generate fusion competence. Secretion can be initiated by only transiently raising the Ca^{2+}, and continues for more than 10 min even in the presence of Ca^{2+} chelators [15]. The fusogen produced in the Ca^{2+}-dependent step may persist under these conditions for a prolonged period of time, the actual fusion of the chromaffin granules being Ca^{2+}-independent.

Inhibitors of phospholipase A_2, an enzyme liberating fatty acids from membrane phospholipids, were shown to be potent inhibitors of catecholamine secretion in both intact and permeabilized cells [24, 25]. As stimulation of cells liberates fatty acids concomitant with or even prior to secretion [25], a phospholipase could be one of the relevant intracellular Ca^{2+} targets. Products of Ca^{2+}-dependent phospholipases, lyso-phospholipids and free fatty acids or some of their metabolites, may be the fusogenic compounds. In a set of *in vitro* experiments we recently found that chromaffin granules fuse with target membrane vesicles even in the absence of Ca^{2+}, provided these vesicles contain fusogenic components previously liberated from membrane phospholipids [26]. *In vitro*, the fusogen is stable in the target membranes in the absence of free Ca^{2+}, as it is in the permeabilized PC 12 and chromaffin cells mentioned above. These experiments do not exclude that Ca^{2+} may be involved in several other processes contributing to secretion. One of these may include the Ca^{2+}-binding protein calpactin, which has been shown to fuse chromaffin granule membranes *in vitro* when arachidonic acid is present [27]. Calpactin is also able to restore secretory activity partially lost in permeabilized cells after prolonged incubation [28]. Addition of a consensus peptide found in a family of Ca^{2+}-binding proteins including calpactin [29] partially inhibits secretion. These results suggest a role for calpactin, together with Ca^{2+} and fatty acids, in the fusion process.

Extensive secretory activity observed in permeabilized or in heavily stimulated intact cells could be the result of unrestrained activation of fusogenic compounds due to the general elevation of the free Ca^{2+} concentration throughout the cell. In contrast, short stimulation of intact cells may transiently activate fusogens in restricted regions of Ca^{2+} influx. Termination of secretion could be regulated by degradation of the fusogen or by its dilution through diffusion.

In this context it seems worthwhile to recall a publication [30], dating back almost to the introduction of the term "stimulus–secretion coupling" [31]. It reports the release of prostaglandin, a metabolite of arachidonic acid, from the perfused adrenal gland upon stimulation by the splanchnic nerve. Unlike the role of Ca^{2+}, the involvement of prostaglandins in secretion was not followed up until recently [32].

Fusion and restoration. To investigate the mechanism of the actual membrane fusion, liposomes and planar lipid bilayers are used as model systems. Despite many efforts, the fusion process is still far from understood (for a recent concept, see Ref. 33). It is also not clear whether these models mimic fusion in biological systems (for discussion, see Ref 34). Promising results come from the application of the patch-clamp technique which allows the investigation of exocytotic fusion processes in single cells with high temporal resolution [35, 36]. It could be demonstrated that in mast cells of the beige mouse the exocytotic membrane fusion of giant secretory ves-

icles with the plasma membrane is initiated by the formation of a fusion pore through both membrane bilayers. The initial pore can open and close in a flickering manner over several milliseconds before expanding into a larger opening of the secretory vesicles towards the extracellular medium [37, 38]. The size of the initial pore can be estimated from conductance measurements to be approximately 1 nm (230 picoSiemens). Interestingly, formation of a pore initiating the fusion also has been reported in a completely different system, the fusion of erythrocyte ghosts with fibroblasts transformed with a viral fusion protein [39]. It suggests the existence of a general principle in biological membrane fusion processes, although the molecular basis for pore formation, e.g. pure lipid conformation changes or pore forming proteins present on both membranes, remains to be established. It will be interesting to examine the putative docking complex composed of chromaffin granule and plasma membrane constituents for the presence of molecules capable of forming such an initial fusion pore. The protein synaptophysin, present on clear synaptic vesicles of brain synaptosomes, has been shown to form a pore when reconstituted in liposomes [40]. The presence of this protein on the large dense-core chromaffin granules, however, is still a matter of dispute, perhaps due to the small number of synaptophysin molecules per chromaffin granule. The controversy may be resolved only when a counterpart of synaptophysin on the plasma membrane can be identified functionally. Expansion of the hypothetical fusion pore results in the incorporation of the chromaffin granule membrane into the plasma membrane. The specific granule membrane proteins are restricted from lateral diffusion by an unknown mechanism which allows the cell to take up the granule membrane by endocytosis, and to recycle it in a next round of secretion [41].

To permit a prompt response to further stimulation, the cell has to re-establish the original status immediately after or even during exocytosis. This implies buffering of the elevated Ca^{2+} concentrations, transport of granules to the periphery, reorganization of the microfilament network, and redocking of granules. These processes may be regulated by a number of different second messengers produced upon activation of various receptors, membrane depolarization and Ca^{2+} influx. The involvement and functional role of phosphatidylinositol turnover, cAMP formation, protein kinase C and GTP-regulatory proteins during exocytosis and the restoration process are only beginning to be unravelled [42].

Physiological relevance of secretory activity measured in vitro

When stimulated, permeabilized cells secrete high proportions, in some reports more than 50%, of the stored hormone. Most likely this is the result of the strength and duration of the stimulus applied, i.e. the flooding of the cells with high concentrations of free Ca^{2+} during many minutes. Secretion may also be augmented by loss or inactivation of desensitizing or inhibitory mechanisms. As similarly high proportions of catecholamines can be secreted by cultured intact cells stimulated with acetylcholine, the physiological significance of such massive secretion has not been questioned. Such over-exposure of the secretory machinery to Ca^{2+} probably does not happen *in vivo*, where secretion is triggered by short depolarizations leading to transient Ca^{2+} influx and, therefore, may explain the discrepancy between the amounts of catecholamines secreted in permeabilized cells and in the adrenal gland *in vivo*.

In adult rats, 40–50 μg of adrenaline is stored in the adrenals [43], whereas the plasma level of adrenaline is about 150 pg/mL under resting conditions [44]. With less than 5 ng of adrenaline in the circulation, the ratio of stored versus plasma adrenaline is about 10,000 to 1. Under stress situations, increasing in severity from handling of animals to immobilization and decapitation, plasma levels of adrenaline increase up to 100-fold [45]. As the effect of the various treatments on adrenaline clearance rates has not been determined, it is difficult to estimate the amount of adrenaline which has to be secreted for establishing the given plasma level. An upper limit for secretion under stress can be obtained from experiments performed on isolated rat adrenal glands. The adrenals can be removed with intact splanchnic innervation, and the adrenaline secreted upon stimulation by the nerve or by exogenously added acetylcholine can be determined in the perfusate [46]. Secretion of adrenaline in the unstimulated gland was about 12 ng in 10 min. By maximal electrical stimulation at 10 Hz for 10 min, this value increased to about 300 ng [47], by stimulation with 20 μM acetylcholine to about 400 ng, and by continuous depolarization by high K^+ to about 1 μg [48]. Since the total adrenaline content of an adrenal medulla is about 20 μg, maximal nerve stimulation leads to the secretion of 1–2% of the stored hormone over 10 min, and even continuous depolarization releases only 5%. Such rates of secretion thus seem to be sufficient to increase the plasma level measured even under acute maximal stress.

The 5- to 10-fold increase in adrenaline plasma levels in response to various types of chronic stress can probably be maintained by the secretion of less than 1% of the stored adrenaline. Since a rat chromaffin cell contains about 30,000 chromaffin granules [49], this corresponds to the fusion of less than 300 granules. The results from stimulated isolated glands are in good agreement with the *in vivo* measurement of increases in adrenaline secretion by stimulation of the splanchnic nerve in conscious calves [50]. The rate of *in vivo* secretion is thus much smaller than what is generally measured from cultured cells *in vitro*. Secretory activity in the physiological range of less than 1% of the stored adrenaline cannot be measured reliably *in vitro*, neither from intact nor from permeabilized cell populations. Nevertheless these *in vitro* systems still are very convenient to screen molecules supposedly involved in secretion. To study the exocytotic process of chromaffin cells under physiological conditions, a more sensitive technique and a more subtle way of stimulation have to be utilized.

Fast secretory responses detected by capacitance measurements

Stimulation of chromaffin cells in response to acetylcholine released from the splanchnic nerve predictably only triggers a brief and transient influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. *In vitro*, this physiological sequence can be mimicked by clamping the voltage of the cell membrane at a hyperpolarized value and switching briefly to a depolarized state to allow for transient influx of Ca^{2+}. Conventional voltage-clamp techniques are based on impaling the cell with microelectrodes. For small cells such as chromaffin cells, the patch-clamp technique in the whole cell configuration can be used. A very tight seal is formed between the cell membrane and a fire-polished glass pipette, and the piece of membrane below the pipette is ruptured to gain electrical and physical access to the cell interior [51]. This allows one to control the membrane potential, to measure cell membrane currents (e.g. inward Ca^{2+} currents), and to monitor the exocytotic activity of this individual cell using concurrent capacitance measurements [35, 52, 53]. The cell membrane capacitance is proportional to the cell surface area, which increases during exocytotic incorporation of vesicle membranes into the plasma membrane. As biological membranes all have a similar specific capacitance of about 1 $\mu F/cm^2$, the incorporation of a single chromaffin granule with a diameter of about 200 nm will increase the cell membrane capacitance by about 1 fF (10^{-15} Farad). The total membrane capacitance of the chromaffin cell is about 5–10 pF ($5–10 \times 10^{-2}$ Farad) [35]. Changes of 1 fF corresponding to the fusion of single chromaffin granules fall just below the detection limit of the measurement.

This approach to the study of exocytosis allows a very brief (millisecond) stimulation, a time resolution of exocytotic activity in the millisecond range, and the unambiguous detection of the fusion of even a few chromaffin granules corresponding to secretion of less than 0.1% of the stored catecholamines [35, 54]. In addition, the solutions on both sides of the cell membrane can be manipulated independently without losing ionic gradients or the polarization of the membrane. Depolarizing voltage pulses as short as 10 msec elicit Ca^{2+} influx causing fast, step-like capacitance increases with an amplitude corresponding to the fusion of less than 100 chromaffin granules. After depolarization there is no further increase in capacitance, indicating that the exocytotic fusion process is completed within milliseconds. This reveals an exquisitely fast stimulus–secretion coupling in chromaffin cells. The step-like capacitance increases require the influx of external Ca^{2+} and their magnitude depends on the amount of Ca^{2+} that enters during depolarization [54, 55]. It is attractive to suggest that these fast capacitance increases reflect the fusion of chromaffin granules docked to the plasma membrane.

A functional involvement of the plasma membrane 51 kD chromaffin granule binding protein in exocytosis has been proven by exploiting this technique. When antibodies or F_{ab}-fragments against this conjectural docking protein are introduced into the cytoplasm through the patch pipette, secretion is inhibited without affecting the Ca^{2+} currents [19]. The existence of docking sites for chromaffin granules on the plasma membrane is strongly implied by these inhibition experiments. Simultaneous measurement of Ca^{2+} currents and exocytosis also reveals an influence of the calmodulin inhibitor trifluoperazine on Ca^{2+} channels and a later step of exocytosis [55]. The patch-clamp technique has also been applied successfully to test the influence on secretion of tetanus toxin. In chromaffin cells, which in contrast to neurons do not have surface receptors for this toxin, tetanus toxin injected into the cytoplasm via the patch pipette completely blocks exocytosis induced by elevation of Ca^{2+} to the micromolar level [56]. Surprisingly, this elegant experimental system has not been used in chromaffin cells to determine the role of second messenger systems other than Ca^{2+} in the regulation of exocytotic fusion. In the non-excitable mast cells, however, the capacitance measurement has been exploited to define multiple signalling pathways [57, 58].

An intriguing aspect of the secretory response has been documented recently in isolated nerve endings from the neurohypophysis by capacitance measurements. Previous experiments had shown that the amounts of hormones secreted from these nerve terminals depends not only on the strength but also on the frequency of stimulation [59]. Substantiating these findings, the capacitance responses to depolarization measured in individual terminals are not determined solely by the magnitude of the Ca^{2+} influx but vary with the pattern of stimulation [60]. The real-time, high sensitivity measurement of exocytosis thus offers the possibility to investigate directly the transduction of complex stimulatory patterns into intricate secretory responses such as facilitation, potentiation, or desensitization.

Summary and outlook

Intact and permeabilized chromaffin cells have been used successfully to distinguish molecules involved in exocytosis, albeit their functional role in secretion could not yet be characterized. The inevitable over-stimulation by prolonged exposure to high concentrations of secretagogues has made it difficult to assess the physiological significance of these molecules. Stimulation of the intact perfused gland by the splanchnic nerve represents a physiologically more relevant experimental situation, at least for measuring the influence of extracellularly administered molecules. But it is not suited to study individual cells nor to reach the intracellular compartment with molecular probes.

The patch-clamp technique in conjunction with capacitance measurements offers succour. Rigorous analysis of the step capacitance increases will allow the dissection of exocytosis into various steps such as transport, docking and fusion through their manipulation by proper control of both the extra- and the intracellular compartments. The stimulatory or inhibitory influence of extracellularly added ligands on secretion can be tested separately from, or concomitantly with, alterations induced in the intracellular second messenger systems. The chromaffin cells can be viewed as giant presynaptic nerve

terminals offering many advantages over other preparations. They represent an almost homogeneous population of cells and are easily isolated in large amounts required for biochemical studies. The discovery of fast excitation–secretion coupling has made the chromaffin cell an even more attractive model system to study neurotransmitter secretion.

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REFERENCES

1. Baker PF and Knight DE, Calcium-dependent exocytosis in bovine adrenal medullary cells with leaky plasma membranes. *Nature* **276**: 620–622, 1978.
2. Dunn LA and Holz RW, Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J Biol Chem* **253**: 4989–4993, 1983.
3. Wilson SP and Kirshner N, Calcium-evoked secretion from digitonin-permeabilized adrenal medullary chromaffin cells. *J Biol Chem* **258**: 4994–5000, 1983.
4. Bader M-F, Thiersé D, Aunis D, Ahnert-Hilger G and Gratzl M, Characterization of hormone and protein release from α-toxin-permeabilized chromaffin cells in primary culture. *J Biol Chem* **261**: 5777–5783, 1986.
5. Schäfer Th, Karli UO, Gratwohl EK-M, Schweizer FE and Burger MM, Digitonin-permeabilized cells are exocytosis competent. *J Neurochem* **49**: 1697–1707, 1987.
6. Cooke P and Poisner AM, Microfilaments in bovine adrenal chromaffin cells. *Cytobiologie* **13**: 442–450, 1976.
7. Cheek TR and Burgoyne RD, Nicotine-evoked disassembly of cortical filaments in adrenal chromaffin cells. *FEBS Lett* **207**: 110–114, 1986.
8. Cheek TR and Burgoyne RD, Cyclic AMP inhibits both nicotine-induced actin disassembly and catecholamine secretion from bovine adrenal chromaffin cells. *J Cell Biol* **262**: 11663–11666, 1987.
9. Perrin D and Aunis D, Reorganization of α-fodrin induced by stimulation in secretory cells. *Nature* **315**: 589–592, 1985.
10. Perrin D, Langley OK and Aunis D, Anti-α-fodrin inhibits secretion from permeabilized chromaffin cells. *Nature* **326**: 498–501, 1987.
11. Cheek TR, Jackson TR, O'Sullivan AJ, Moreton RB, Berridge MJ and Burgoyne RD, Simultaneous measurements of cytosolic calcium and secretion in single bovine adrenal chromaffin cells by fluorescent imaging of fura-2 in cocultured cells. *J Cell Biol* **109**: 1219–1227, 1989.
12. Heuser JE, Reese TS, Dennis MJ, Jan Y, Jan L and Evans L, Synaptic vesicle exocytes captured by quick freezing and correlated with quantal transmitter release. *J Cell Biol* **81**: 275–300, 1979.
13. Landis DMD, Hall AK, Weinstein LA and Reese TS, The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. *Neuron* **1**: 201–209, 1988.
14. Plattner H, Synchronous exocytosis in *Paramecium* cells. In: *Cell Fusion* (Ed. Sowers E), pp. 69–98. Plenum Press, New York, 1987.
15. Schäfer T, Karli UO, Schweizer FE and Burger MM, Docking of chromaffin granules—A necessary step in exocytosis? *Biosci Rep* **7**: 269–279, 1987.
16. Nakata T, Sobue K and Hirokawa N, Conformational change and localization of calpactin I complex involved in exocytosis as revealed by quick-freeze, deep-etch electron microscopy and immunocytochemistry. *J Cell Biol* **110**: 13–25, 1990.
17. Palade G, Intracellular aspects of the process of protein synthesis. *Science* **189**: 347–358, 1975.
18. Meyer D and Burger MM, Isolation of a protein from the plasma membrane of adrenal medulla which binds to secretory vesicles. *J Biol Chem* **254**: 9854–9859, 1979.
19. Schweizer FE, Schäfer T, Tapparelli C, Grob M, Karli UO, Heumann R, Thoenen H, Bookman RJ and Burger MM, Inhibition of exocytosis by intracellularly applied antibodies against a chromaffin granule binding protein. *Nature* **339**: 709–712, 1989.
20. Hochner B, Parnas H and Parnas I, Membrane depolarization evokes neurotransmitter release in the absence of calcium entry. *Nature* **342**: 433–435, 1989.
21. Burgoyne RD, The relationship between secretion and intracellular free calcium in bovine adrenal chromaffin cells. *Biosci Rep* **4**: 605–611, 1984.
22. Penner R and Neher E, The role of calcium in stimulus–secretion coupling in excitable and non-excitable cells. *J Exp Biol* **139**: 329–345, 1988.
23. Rosenheck K and Plattner H, Ultrastructural and cytochemical characterization of adrenal medullary plasma membrane vesicles and their interaction with chromaffin granules. *Biochim Biophys Acta* **856**: 373–382, 1986.
24. Frye RA and Holz RW, Phospholipase A_2 inhibitors block catecholamine secretion and calcium uptake in cultured bovine adrenal chromaffin cells. *Mol Pharmacol* **23**: 547–550, 1983.
25. Frey RA and Holz RW, Arachidonic acid release and catecholamine secretion from digitonin-permeabilized chromaffin cells: Effects of micromolar calcium, phorbol ester, and protein alkylating agents. *J Neurochem* **44**: 265–273, 1985.
26. Karli UO, Schäfer T and Burger MM, Fusion of neurotransmitter vesicles with target membrane is calcium independent in a cell-free system. *Proc Natl Acad Sci USA*, in press.
27. Drust DS and Creutz CE, Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. *Nature* **331**: 88–91, 1988.
28. Ali SM, Geisow MJ and Burgoyne RD, A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. *Nature* **340**: 313–315, 1989.
29. Geisow MJ, Fritsche U, Hexham JM, Dash B and Johnson T, A consensus amino-acid sequence repeat in *Torpedo* and mammalian Ca^{2+}-dependent membrane-binding proteins. *Nature* **320**: 636–638, 1986.
30. Ramwell PW, Shaw JE, Douglas WW and Poisner AM, Efflux of prostaglandin from adrenal glands stimulated with acetylcholine. *Nature* **210**: 273–274, 1966.
31. Douglas WW and Rubin RP, The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J Physiol (Lond)* **159**: 40–57, 1961.
32. Moskovitz N, Schook W and Puszkin S, Interaction of brain synaptic vesicles induced by endogenous Ca^{2+}-dependent phospholipase A_2. *Science* **216**: 305–307, 1982.
33. Helm CA, Israelachvili JN and McGuiggan PM, Molecular mechanisms and forces involved in the adhesion and fusion of amphiphilic bilayers. *Science* **246**: 919–922, 1989.
34. Plattner H, Regulation of membrane fusion during exocytosis. *Int Rev Cytol* **119**: 197–286, 1989.
35. Neher E and Marty A, Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc Natl Acad Sci USA* **79**: 6712–6716, 1982.
36. Penner R and Neher E, The patch-clamp technique in the study of secretion. *Trends Neurosci* **12**: 159–163, 1989.
37. Zimmerberg J, Curran M, Cohen FS and Brodwick M, Simultaneous electrical and optical measurements show

that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. *Proc Natl Acad Sci USA* **84**: 1585–1589, 1987.
38. Breckenridge LJ and Almers W, Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature* **328**: 814–817, 1987.
39. Spruce AE, Iwata A, White JM and Almers W, Patch clamp studies of single cell-fusion events mediated by a viral fusion protein. *Nature* **342**: 555–558, 1989.
40. Thomas L, Hartung K, Langosch D, Rehm H, Bamberg E, Franke WW and Betz H, Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science* **242**: 1050–1053, 1988.
41. Patzak A and Winkler H, Exocytotic exposure and recycling of membrane antigens of chromaffin cells: Ultrastructural evaluation after immunolabeling. *J Cell Biol* **102**: 510–515, 1986.
42. Augustine GJ, Charlton MP and Smith SJ, Calcium action in synaptic transmitter release. *Annu Rev Neurosci* **10**: 633–693, 1987.
43. Howe PRC, Rogers PF, Morris MJ, Chalmers JP and Smith RM, Plasma catecholamines and neuropeptide-Y as indices of sympathetic nerve activity in normotensive and stroke-prone spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **8**: 1113–1121, 1986.
44. Schober M, Howe PR, Sperk G, Fischer-Colbrie R and Winkler H, An increased pool of secretory hormones and peptides in adrenal medulla of stroke-prone spontaneously hypertensive rats. *Hypertension* **13**: 469–474, 1989.
45. Popper CW, Chiueh CC and Kopin IJ, Plasma catecholamine concentrations in unanesthetized rats during sleep, wakefulness, immobilization and after decapitation. *J Pharmacol Exp Ther* **202**: 144–148, 1977.
46. Wakade AR, Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal medulla. *J Physiol (Lond)* **313**: 463–480, 1981.
47. Malhotra RK, Wakade TD and Wakade AR, Cross-communication between acetylcholine and VIP in controlling catecholamine secretion by affecting cAMP, inositol trisphosphate, protein kinase C, and calcium in rat adrenal medulla. *J Neurosci* **9**: 4150–4157, 1989.
48. Malhotra RK, Wakade TD and Wakade AR, Comparison of secretion of catecholamines from the rat adrenal medulla during continuous exposure to nicotine, muscarine or excess K. *Neuroscience* **26**: 313–320, 1988.
49. Nordmann JJ, Combined stereological and biochemical analysis of storage and release of catecholamines in the adrenal medulla of the rat. *J Neurochem* **42**: 434–437, 1984.
50. Edwards AV, Adrenal catecholamine output in response to stimulation of the splanchnic nerve in bursts in the conscious calf. *J Physiol (Lond)* **327**: 409–419, 1982.
51. Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* **391**: 85–100, 1981.
52. Joshi C and Fernandez JM, Capacitance measurements: An analysis of the phase detector technique used to study exocytosis and endocytosis. *Biophys J* **53**: 885–892, 1988.
53. Lindau M and Neher E, Patch-clamp techniques for time-resolved capacitance measurements in single cells. *Pflügers Arch* **411**: 137–146, 1988.
54. Bookman RJ and Schweizer F, Fast and slow phases in chromaffin cell exocytosis depend on Ca entry and Ca buffering. *J Gen Physiol* **92**: 4a, 1988.
55. Clapham DE and Neher E, Trifluoperazine reduces inward ionic currents and secretion by separate mechanisms in bovine chromaffin cells. *J Physiol (Lond)* **353**: 541–564, 1984.
56. Penner R, Neher E and Dreyer F, Intracellular injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature* **324**: 76–78, 1986.
57. Penner R, Multiple signaling pathways control stimulus–secretion coupling in rat peritoneal mast cells. *Proc Natl Acad Sci USA* **85**: 9856–9860, 1988.
58. Penner R and Neher E, Regulation of calcium influx by second messengers in rat mast cells. *Nature* **334**: 499–504, 1988.
59. Nordmann JJ and Stuenkel EL, Electrical properties of axons and neurohypophysial nerve terminals and their relationship to secretion in the rat. *J Physiol (Lond)* **389**: 521–539, 1986.
60. Fidler LinN, Nowycky MC and Bookman RJ, Direct measurements of exocytosis and calcium currents in single isolated nerve terminals. *Nature* **344**: 449–451, 1990.