The patch-clamp technique in the study of secretion

Reinhold Penner and Erwin Neher

One of the basic cellular functions of virtually every cell type is the exocytotic release of molecules synthesized, stored and packaged into intracellular vesicles or granules. Over decades much effort has been concentrated on elucidating the chain of events leading to exocytosis. Unfortunately, the nature of the process that ultimately induces membrane fusion is not known, nor has it been established definitively whether or not the final steps in the secretory cascade are identical in different cells. Although the fusion between vesicle and plasma membrane has been neatly documented by electron micrographs, it was only recently that the technique of time-resolved membrane capacitance measurement has provided a more detailed insight into mechanistic aspects of exocytosis, both in terms of the fusion event and the steps involved in stimulus–secretion coupling.

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The concentration of free intracellular calcium $[Ca^{2+}]_i$ has long been recognized as the most important regulator of secretory processes[1,2]. Recently, however, a number of signal pathways have been invoked to 'modulate' transmitter release. Figure 1 summarizes and lists some of the molecular mechanisms that have been discussed (see Ref. 3 for review). Apart from Ca^{2+}, the most obvious mechanism is the dual signal pathway[4], resulting in the breakdown of polyphosphoinositides and supplying the two second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). The latter in particular, or its substitute phorbol ester, has been shown to augment Ca^{2+}-mediated secretion at the endplate[5], in chromaffin cells[6] and in neurones[7]. In non-neuronal cells, secretion can be elicited this way even in the absence of Ca^{2+} (Refs 8–10). Molecular mechanisms discussed as mediators of such modulation include phosphorylation of proteins involved in exocytosis or vesicle processing[11], osmotic swelling of vesicles after activation of Ca^{2+}-activated channels in the vesicle membrane[12], generation of lysolipids and arachidonic acid by phospholipase A_2 (Ref. 13) or direct influence on the exocytotic process by specialized GTP-binding proteins, termed G_e (Ref. 14). Furthermore, interactions between the cytoskeleton and vesicles have been established as sites for modulating control of secretion[11,15].

The patch-clamp technique offers a number of tools to study the signal pathways involved. First, it allows the recording of single channels and whole-cell currents that are involved in the regulation of $[Ca^{2+}]_i$. Second, in favourable cases it provides an assay for the secretory process itself through membrane capacitance measurements (see Fig. 2). The technique has such high-quality resolution that fusion events of single vesicles can be captured (see Fig. 1). Third, it is readily combined with the measurement of $[Ca^{2+}]_i$ by the Ca^{2+} indicator dye, fura-2[16] (Fig. 2); and finally, it allows 'dialysation' of the cell interior with solutions of known composition since there is rapid diffusional exchange between a patch pipette and a small cell in the tight-seal, whole-cell recording mode[17]. This, of course, is an ambiguous tool: on the one hand it supplies substances of interest such as second messengers, regulatory proteins and molecular probes to the cell interior (Fig. 1, see Ref. 18 for review); on the other, it inevitably leads to the washout of endogenous substances. Such washout has been known for a long time in connection with Ca^{2+} channels[19]. The process of exocytosis turned out to be particularly sensitive to it[20].

In this review we summarize the role of these tools in the recent progress in the study of exocytosis. Partly for technical reasons, a great deal of the material covered is derived from peritoneal mast cells, a cell type particularly suitable for the tools described above. Mast cells secrete histamine and other chemical mediators in response to a variety of stimuli[21]. Although they behave in a somewhat 'non-classical' way, particularly with respect to Ca^{2+} (see Fig. 2), they may well turn out to be a good model for non-Ca^{2+} modulatory control mechanisms (see below).

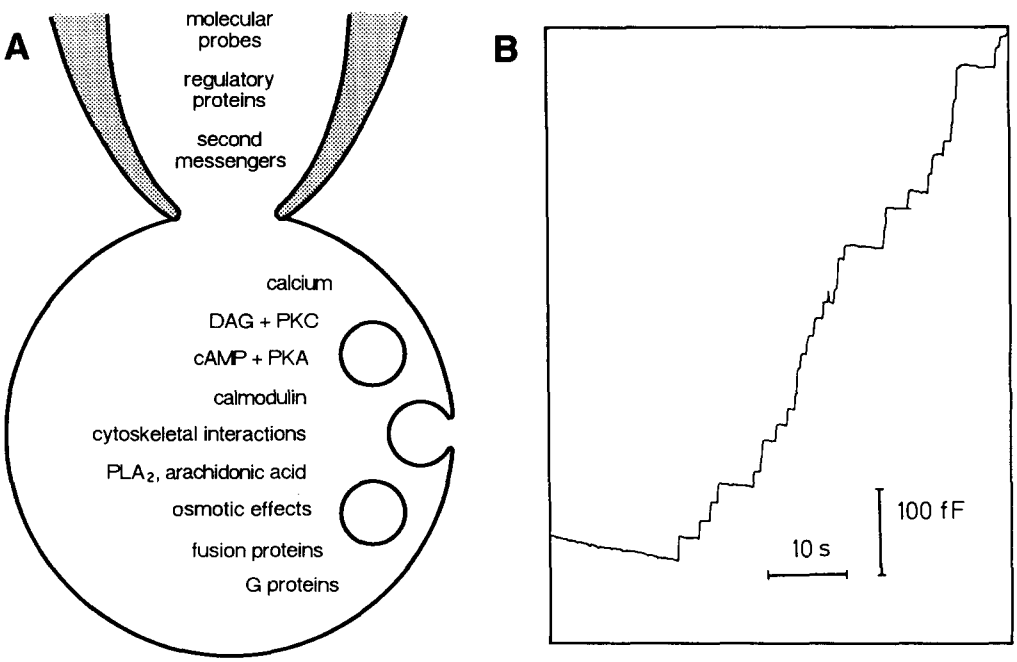

Fig. 1. **(A)** *Schematic diagram of a cell in the whole-cell configuration of the patch-clamp technique, indicating the potential use of dialysing the cytoplasm with solutions of desired composition. Some of the mechanisms discussed as modulators of exocytotic vesicle fusion are listed.* **(B)** *Step-wise changes of cell membrane capacitance following fusion events of single granules, recorded in a mast cell stimulated with GTP-γ-S. Changes in capacitance correspond to increases of the cell surface area, due to the incorporation of the granule membrane into the plasma membrane during exocytosis.* (Taken, with permission, from Ref. 34.)

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Membrane capacitance as an assay for exocytosis

Implicit in the process of exocytosis is the incorporation of vesicle membranes into the plasma membrane. As a result, the total cell surface area is bound to increase concomitant with secretory activity, depending on the number of fusing vesicles and provided the rate of secretion exceeds the rate of endocytotic membrane retrieval. Therefore, one may obtain an estimate of the secretory activity of a single cell by quantifying the cell membrane capacitance[22–24] (see Fig. 2), which is proportional to membrane surface area. Depending on the cell type, the available equipment and the desired resolution, various methods have been used to assess membrane capacitance: (1) the pseudo-random binary sequence (PRBS) technique uses the transfer function of a given stimulation to obtain membrane capacitance[25]; (2) the time-domain technique determines capacitance from the current relaxation in response to a voltage step[26]; (3) the two-phase lock-in technique derives passive membrane parameters by delivering a sinusoidal voltage command and measuring the resulting current response at two orthogonal phases[24,26]. Of these, the latter has gained most acceptance since its resolution is highest and it allows capacitance to be monitored in real time.

Capacitance measurements have been applied to a number of different cell types (see Fig. 2) including chromaffin cells[24,27], PC-12 cells (Penner, R. and Neher, E., unpublished observations), mast cells[28,29], β-cells[29], neutrophils[30], oocytes[31], lactotrophs[32] and pancreatic acinar cells[33]. However, it should be borne in mind that capacitance measurements may not be applicable to all secretory systems. For example, we have been unable to detect significant increases in membrane capacitance to various stimuli in parotid cells, rat basophilic leukaemia cells or hepatocytes. So

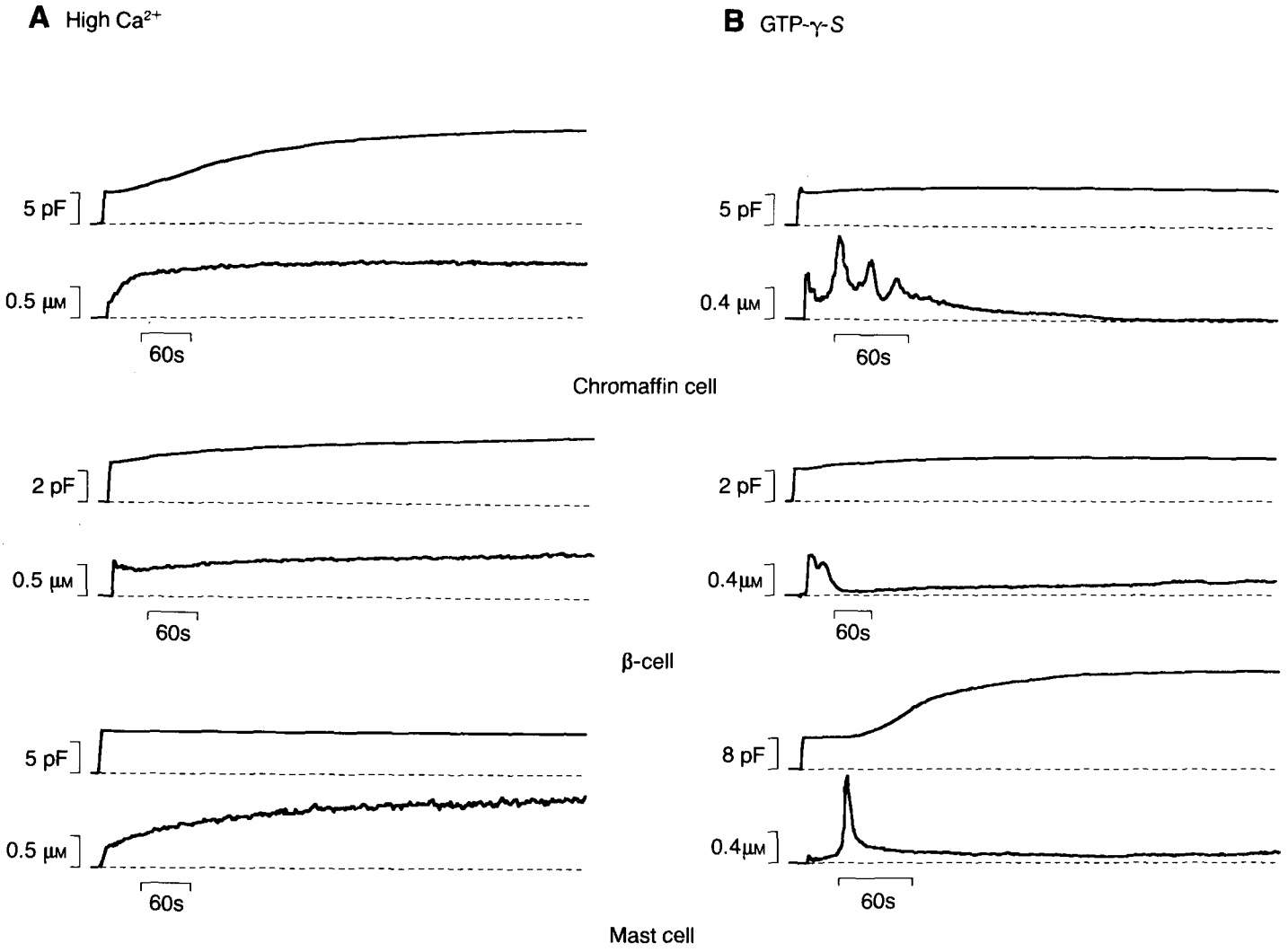

Fig. 2. *Secretory responses of different cell types stimulated by injection of micromolar intracellular calcium concentrations ($[Ca^{2+}]_i$) or GTP-γ-S. Fura-2 was used to monitor $[Ca^{2+}]_i$ and changes in cell membrane capacitance reflect increases in membrane area as a result of exocytosis. Note that the resolution of capacitance changes is lower here than in Fig. 1, in order to monitor the time course and the total extent of secretion in a single cell.* **(A)** *Cells were dialysed with pipettes containing standard intracellular solutions supplemented with mixtures of Ca^{2+}-EGTA/EGTA that clamped $[Ca^{2+}]_i$ at about 1 μM.* **(B)** *Secretory responses and typical changes in $[Ca^{2+}]_i$ of different cell types stimulated by injection of GTP-γ-S. Cells were stimulated with 40–100 μM GTP-γ-S added to the standard internal solution. From these results it is clear that chromaffin cells secrete in response to elevated $[Ca^{2+}]_i$ but not to GTP-γ-S, whereas the opposite is true for mast cells. Interestingly, β-cells can respond to both stimuli. The smaller amplitudes of secretory responses of β-cells as compared with chromaffin cells and mast cells may result from differences in the number of vesicles contained in these cells or from a different ratio of exocytotic to endocytotic activity.* (Taken, with permission, from Ref. 29.)

far, those cell types that have been investigated successfully contain many vesicles and release most of them within a short period of time. This yields a large secretory response that can be quantified. Other cell types are known to secrete slowly over a period of tens of minutes and even though secretion may proceed steadily, it may not show up as a capacitance increase if concomitant endocytic membrane retrieval balances the vesicle membranes added by exocytosis. Decreases in capacitance, probably due to endocytosis, have been observed in mast cells[34] and chromaffin cells[27]. Another drawback to the application of capacitance measurements may arise from complicated morphology of the cells or the tissue to be investigated. Quantitative capacitance measurements are based on assumptions such as the uniform specific capacitance of the various membranes involved and the compact shape of cells. Thus, electrical coupling between cells or extensive cell processes may prevent an accurate assessment of membrane capacitance. Since changes in conductance, series resistance and other imponderables can develop during an experiment, much caution must be taken in the analysis of the data. Also, analysis has to be restricted to a voltage range in which a given cell behaves 'passively', i.e. no voltage-dependent conductances should be activated in this range. Nevertheless, a number of important questions regarding various aspects of exocytosis could be answered by using capacitance measurements. Some of the main findings are addressed below.

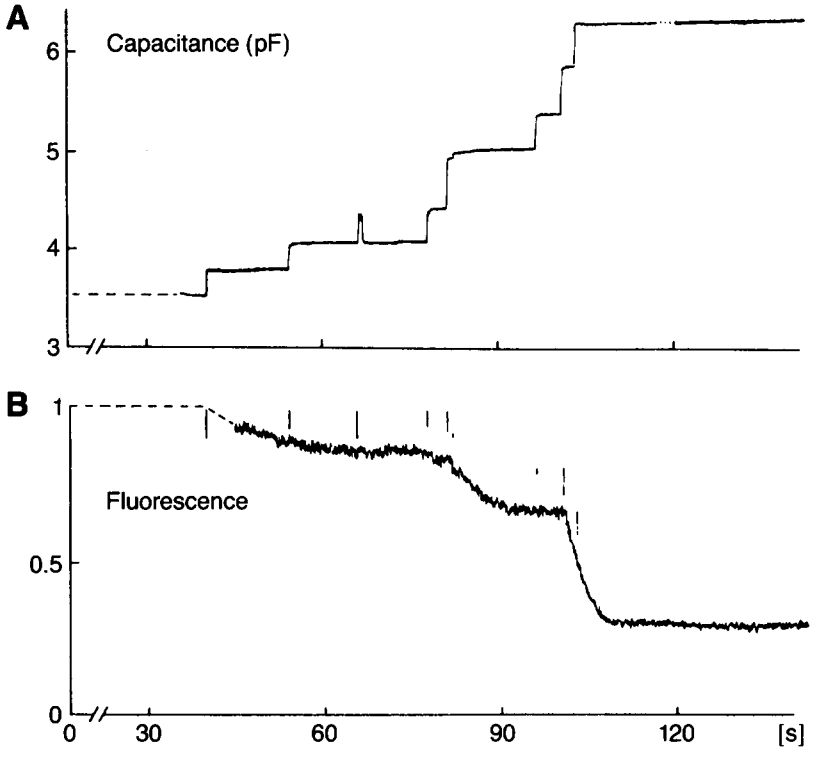

Fig. 3. *Capacitance and fluorescence changes in mast cells from beige mice.* **(A)** *Capacitance increases due to fusion of 'giant' granules.* **(B)** *To demonstrate the release of granule contents, cells were incubated with quinacrine in order to trap the dye in the granules and monitor the loss of fluorescence following fusion.* (Taken, with permission, from Ref. 37.)

The fusion event

Neher and Marty[24] refined capacitance measurement to the point that discrete changes in membrane capacitance could be observed during Ca^{2+}-induced secretion in bovine adrenal chromaffin cells. They resolved small capacitance steps (on-steps) of about 1 fF (10^{-15} farad), which accord with the average surface areas of chromaffin granules as determined from morphometric data assuming a specific capacitance of 1 $\mu F/cm^2$. In addition, decreases in capacitance (off-steps) were observed, presumably resulting from endocytic membrane retrieval or pinched-off blebs. A similar correlation of the amplitude of step-wise changes in capacitance with morphometric size distributions of vesicles was found in mast cells[28] and neutrophils[30], suggesting that the technique does resolve single fusion events. In fact, the technique has been used to study developmental processes of mast cell vesicle maturation[35]. It was found that the distribution of capacitance step sizes shows discrete peaks of fixed increments, suggesting that mast cell granules are composed of fused 'unitary' vesicles.

Mast cells from beige mice may represent an extreme case of this intracellular fusion process, since they possess only ten to 20 giant granules, and this has been taken advantage of in the study of the exocytotic fusion process. It was found that the fusion process (monitored by the capacitance increase) is accompanied by a current transient that reflects the opening of a 'fusion pore'[36,37]. The current through the pore represents the charging or discharging of the vesicles' capacitance as plasma and granule membranes become continuous; the minimum size of this pore was determined to be about 230 pS. Admittance measurements indicate that the pore conductance increases suggesting that the pore dilates to allow release of granule contents as witnessed by the loss of granule-trapped fluorescent dye (see Fig. 3). Close inspection of a number of these fusion events revealed capacitance-flickering (short-lived, repeated capacitance increases with intermittent decreases of the same amplitude), suggesting that the establishment of the fusion pore is not an irreversible step. It was found that it is only after flickering stops that large molecules are released.

These results were interpreted to mean that the fusion process is initiated by the formation of a channel-like structure that may open and close repeatedly before it eventually widens so as to establish continuity between intravesicular and extracellular space. The driving force for the widening of the fusion pore may arise from granule swelling. The swelling of secretory granules was previously discussed as a possible mechanism for the initiation of the fusion process, but simultaneous capacitance and optical measurements showed that membrane fusion precedes granule swelling during exocytosis in beige mouse mast cells[37,38].

Stimulus–secretion coupling

Another important application of capacitance measurements is the investigation of events leading to exocytosis. The simultaneous measurement of $[Ca^{2+}]_i$ and capacitance in a single cell provides a powerful tool to study the role of $[Ca^{2+}]_i$ in secretion in excitable and non-excitable cells. Thus it was shown that an elevation of $[Ca^{2+}]_i$ either by Ca^{2+}-buffered intrapipette solutions or by stimulating Ca^{2+} influx

through voltage-activated Ca^{2+} channels is sufficient to cause secretion in adrenal chromaffin cells[24] or pancreatic β-cells[29] (Fig. 2). There is evidence for two phases of secretion in chromaffin cells, as short stimulation by depolarizing voltage pulses causes a small capacitance increase within 5–50 ms, whereas longer depolarizations induce additional, larger increases after a delay[39]. These two secretion 'modes' may reflect fusion of docked granules following localized increases in $[Ca^{2+}]_i$ beneath the plasma membrane due to a short-lived Ca^{2+} influx and the time-lagged secretion of more remote granules that may require transport to the plasma membrane (which is only effected during sustained elevation of cytosolic $[Ca^{2+}]_i$ in the whole cell).

In contrast to excitable cells, mast cells do not secrete in response to a simple elevation of $[Ca^{2+}]_i$ (Fig. 2), unless unphysiologically high levels are used[40]. Secretion in these cells is initiated following stimulation with antigen, substance P or synthetic polycations such as compound 48/80. Agonist-stimulated secretion is blocked by pertussis toxin or GDP-β-*S*, indicating that cell surface receptors couple to GTP-binding proteins which transduce the extracellular signal into various signalling cascades. Not surprisingly, GTP-γ-*S* can induce secretion in mast cells (but not chromaffin cells), when included in the pipette solution, by irreversibly activating these G proteins[9,28] (Fig. 2). In concordance with data obtained from permeabilized cells[41], GTP is an absolute prerequisite for secretory responses by external agonists and has to be provided with the pipette solution when dialysing mast cells in the whole-cell configuration of the patch-clamp technique[20].

Although mast cell secretagogues effect a transient rise of $[Ca^{2+}]_i$ by activating the 'dual signal pathway' [receptor → G protein (Gp) → phospholipase C → IP_3 and DAG → release of Ca^{2+} from intracellular stores and activation of protein kinase C], the increase in $[Ca^{2+}]_i$ is neither sufficient nor necessary for secretion to occur, since, as mentioned above, an elevation of $[Ca^{2+}]_i$ does not induce secretion by itself and secretagogues can induce exocytosis at a $[Ca^{2+}]_i$ clamped to basal levels[42] or even lower (as is the case with GTP-γ-*S* and 10 mM internal EGTA)[10]. In this respect, permeabilized mast cells in suspension seem to behave differently, as an obligatory synergy between increased Ca^{2+} and cyclic nucleotides has been found[41]. Patch-clamp studies indicate that the role of $[Ca^{2+}]_i$ in mast cell secretion is a modulatory one in that the rate of secretion is enhanced by elevated levels of $[Ca^{2+}]_i$, provided that an additional signal triggers the release process[10]. In fact, mast cells have developed remarkable mechanisms to regulate $[Ca^{2+}]_i$ (Ref. 43). In addition to releasing Ca^{2+} from intracellular stores by IP_3, this same second messenger induces Ca^{2+} influx through a highly Ca^{2+}-selective pathway. Furthermore, an unknown second messenger supports Ca^{2+} influx by activating cation channels permeable to divalent ions. At the same time, the membrane potential of mast cells is clamped to negative values by virtue of a chloride conductance that can be activated by cAMP and $[Ca^{2+}]_i$, and which ensures a considerable electrical driving force for Ca^{2+} entry.

The step that renders secretion sensitive to $[Ca^{2+}]_i$ may result from activation of protein kinase C (PKC). However, full activation of the enzyme by phorbol esters does not induce appreciable secretion at basal $[Ca^{2+}]_i$, although it synergizes with a concomitant rise in $[Ca^{2+}]_i$ (Ref. 44). The question arises, what is the signal that allows mast cells to secrete at basal or even sub-basal levels of $[Ca^{2+}]_i$?

From a number of experiments both biochemical and electrophysiological in which pharmacological tools were used to investigate the various signalling pathways, it was found that the dual signal pathway cannot account entirely for stimulus–secretion coupling in some non-excitable cells.

- Secretion in neutrophils and mast cells can be induced by GTP-γ-*S* in the presence of neomycin[14,45] [which blocks polyphosphoinositide (PI) breakdown] and this suggests that a G protein is involved in exocytosis (G_e).
- Ca^{2+} transients (which may be taken as evidence for PI breakdown and activation of the dual signal pathway) and secretion are differentially affected by 'washout', i.e. the generation of Ca^{2+} transients is not impeded by prolonged intracellular dialysis of cells, whereas secretory responses are rapidly lost due to washout[20].
- Ca^{2+} transients and degranulation do not always show a strict temporal correlation, i.e. in some cells, secretion can start before Ca^{2+} transients are manifest[10].
- Secretory responses following receptor stimulation can be blocked selectively by intracellular cAMP without blocking PI breakdown as shown by the presence of Ca^{2+} transients[46].
- Conversely, GTP-γ-*S* or compound 48/80 can induce secretion without changes of $[Ca^{2+}]_i$ after preincubation of cells with phorbol ester[46] and this effect is probably not caused by the activation of PKC via the dual signal pathway since the enzyme is presumably activated maximally already.

Taken together, these findings clearly point towards an as yet unidentified signalling pathway for exocytosis that is under the control of a G protein tentatively termed G_e and distinct from the G protein that transduces PI breakdown (G_p). Whether this G protein mediates activation of a signalling cascade or controls exocytosis directly is not yet known.

The attempt to address the question whether the mechanisms that govern secretory responses in these non-excitable cells are of any relevance to neurotransmitter release, leaves several speculations. The requirements for vesicular release in excitable and non-excitable cells in terms of speed are inherently different by several orders of magnitude and might, therefore, very well be reflected in different secretory mechanisms. Yet it appears that the various components of secretion control (Ca^{2+} and enzymatic machinery) are present in both excitable and non-excitable cells. From our present limited understanding, it is conceivable that the slower enzymatic steps dominate stimulus–secretion coupling in non-excitable cells, with Ca^{2+} as a modulator, whereas the reverse may be true for excitable cells. Since the latter have to respond fast, the exocytotic machinery may be

poised so that the rate-limiting step is a Ca^{2+}-dependent one, which can be switched on rapidly by electrical activity while slow modulation of transmitter release can still be exerted by the enzymatic steps.

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Acknowledgements
We thank our colleagues for allowing us access to their unpublished material.

book review

Neuromethods Vol. 9: The Neuronal Microenvironment

by Alan A. Boulton, Glen B. Baker and Wolfgang Walz, The Humana Press, 1988. $94.50 in USA ($99.50 elsewhere) (xxvi + 732 pages) ISBN 0 89603 115 2

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This volume covers a neglected area from the unusual perspective of the methods currently in use in the field. Previous volumes in the series have nearly all been concerned with different aspects of neurochemistry; this is the first to take a biological concept (the internal environment of the brain) and present the wide variety of methods used to study it.

What might one hope for in a text on 'Neuromethods'? Presumably, a description of individual methods with enough detail for the unfamiliar to be able to set them up or at least an outline with references to detailed descriptions in the literature. A critical evaluation of the methods, stressing strengths and weaknesses and sources of artefact would be invaluable to the uninitiated (and perhaps also for those who have been in the field so long as to be in danger of overlooking some of the limitations of over-familiar methods). What one would hope not to see would be a lengthy review of an author's own work for which a mention of the methods used was largely incidental. This volume gives examples of the use of techniques for specific problems, which is of course important and useful in giving insight into the range of problems that have been tackled, but in some instances the examples are rather removed from the nervous system, which might leave readers wondering whether the technique would be applicable. Thus the detailed description of 'Patch clamp recording methodology' contains a brief section on cell-isolation procedures but deals with various types of muscle cell and epithelia, rather than nervous tissue.

The approach of authors of individual chapters is so varied that it is difficult to discern any underlying editorial policy. Melton gives a brief but useful evaluation of some of the commoner methods used in blood–brain–cerebrospinal fluid exchange experiments. In fact his coverage is more comprehensive than the title ('Cerebrovascular water and ion transport') suggests. Only an outline of the various methods is given but each is well referenced, so that the reader will be able to find full descriptions of the methods in the literature. His concise evaluations of each method provide a useful summary of the possibilities and limitations of these methods.

Several of the other authors give very detailed descriptions of