

## STIMULUS–SECRETION COUPLING IN EXCITABLE CELLS: A CENTRAL ROLE FOR CALCIUM

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### Summary

Secretion of vesicular contents by exocytosis is a common feature of neuroendocrine secretory cells such as adrenal chromaffin cells and PC12 cells. Although it is clear that in these cells an elevation in intracellular calcium concentration,  $[Ca^{2+}]_i$ , is the triggering event that induces secretion, recent studies using video-imaging, patch-clamp and flash photolysis techniques have all indicated that the  $Ca^{2+}$  signal that triggers secretion is in fact very complex, with the subcellular distribution of  $Ca^{2+}$  being of particular importance along with the magnitude of the rise.

It has become evident that  $Ca^{2+}$  signals with different spatial profiles can be triggered in the same cell by a given stimulus, depending upon the nature of the  $Ca^{2+}$  signalling pathway activated, and that this ability to be able to vary the method of delivery of  $Ca^{2+}$  into the cell is important physiologically, because it provides a means of obtaining differential activation of  $Ca^{2+}$ -dependent processes.

### Introduction

The release of neurotransmitters and hormones by exocytosis is probably the most extensively studied process for establishing the crucial role of  $Ca^{2+}$  as the triggering and controlling event. However, although the concept of  $Ca^{2+}$  control of exocytosis dates back over 20 years (Douglas, 1968), only recently developed methodological approaches have provided a proper quantitative evaluation of the role  $Ca^{2+}$  plays in this process. For example, in adrenal chromaffin cells, the use of video-imaging techniques to visualise the stimulus-induced changes in the concentration of cytosolic free  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) in Fura-2-loaded cells (see reviews by Burgoyne, 1991; Cheek, 1991), and of whole-cell patch-clamp (Augustine and Neher, 1992*a*; Neher and Augustine, 1992) coupled with flash photolysis (Neher and Zucker, 1993) techniques to manipulate  $[Ca^{2+}]_i$  directly at the single-cell level, have indicated that the entry of external  $Ca^{2+}$ , rather than the release of  $Ca^{2+}$  from intracellular stores, is the vital trigger for secretion from these cells, and that this is likely to be because only entry of  $Ca^{2+}$  from the outside medium delivers  $Ca^{2+}$  in sufficient amounts to the subplasmalemmal exocytotic sites.

The mechanisms by which local large increases in  $[Ca^{2+}]_i$  beneath the plasma membrane activate exocytosis and the nature of the components of the exocytotic machinery are now beginning to be resolved, but we still only have limited insight into these aspects. It seems

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likely that the intracellular  $\text{Ca}^{2+}$  receptor mechanism and the exocytotic fusion machinery may involve several cytosolic proteins and membrane proteins, perhaps with varying  $\text{Ca}^{2+}$  affinities, acting in concert (Burgoyne and Morgan, 1993).

This chapter will deal with studies on changes in  $[\text{Ca}^{2+}]_i$  related to exocytosis in electrically excitable cells, and the nature of the  $\text{Ca}^{2+}$  signalling machinery (particularly  $\text{Ca}^{2+}$  channels) involved in the triggering of this process. Considerable emphasis will be given to studies using bovine adrenal chromaffin cells and their clonal counterpart, rat pheochromocytoma PC12 cells, but reference will also be made to relevant studies using other excitable cells.

### Receptor-activated exocytosis in adrenal chromaffin cells

The classical physiological stimulus for catecholamine secretion from bovine adrenal chromaffin cells is acetylcholine acting through cholinergic nicotinic receptors. During the 1980s, however, it became clear that activation of a number of other cell surface receptors either stimulates catecholamine secretion or modulates nicotine-induced secretion (Marley, 1987). Receptor activation in chromaffin cells results either in depolarisation and the opening of voltage-dependent channels in the plasma membrane or in direct activation of phosphoinositidase C, with subsequent generation of the  $\text{Ca}^{2+}$ -mobilising signal inositol 1,4,5-trisphosphate  $[\text{Ins}(1,4,5)\text{P}_3]$ . Nicotine and  $\gamma$ -aminobutyric acid (GABA) lead to depolarisation, whereas muscarinic agonists, bradykinin, angiotensin II, histamine, prostaglandin  $\text{E}_2$ , vasoactive intestinal peptide (VIP) and ATP receptors are all linked to  $\text{Ins}(1,4,5)\text{P}_3$  production *via* activation of phosphoinositidase C. These signalling pathways are not necessarily mutually exclusive because nicotinic stimulation and depolarisation with high  $[\text{K}^+]$  have been reported to lead to  $\text{Ins}(1,4,5)\text{P}_3$  production through  $\text{Ca}^{2+}$ -dependent activation of phosphoinositidase C (Eberhard and Holz, 1988), and  $\text{Ins}(1,4,5)\text{P}_3$  may promote  $\text{Ca}^{2+}$  entry through non-voltage-dependent  $\text{Ca}^{2+}$  channels in the plasma membrane (Mochizuki-Oda *et al.* 1991).

The various receptor agonists differ in their abilities to trigger secretion, although nicotinic stimulation consistently gives the largest response. Apart from nicotinic agonists, only GABA and (after a longer time course) histamine produce a substantial secretory response. Reduced, or indeed no, secretion has been reported in response to the remaining agonists. Irrespective of the nature of the stimulating agent, secretion from intact cells is abolished by the removal of external  $\text{Ca}^{2+}$  (see Burgoyne, 1991), a simple demonstration that  $\text{Ca}^{2+}$  entry and not the release of internally stored  $\text{Ca}^{2+}$  is pivotal in triggering exocytosis from these cells. Indeed, it is by reducing  $\text{Ca}^{2+}$  entry that  $\text{D}_2$  dopamine receptors are thought to exert an inhibitory control on chromaffin cell secretion (Bigornia *et al.* 1988). The importance of  $\text{Ca}^{2+}$  entry *versus* internal  $\text{Ca}^{2+}$  release in triggering secretion is discussed in more detail in the following section.

### The intracellular calcium signal in chromaffin cells

Many laboratories have used  $\text{Ca}^{2+}$ -sensitive fluorescent indicator dyes in intact cells to follow the stimulus-induced changes in  $[\text{Ca}^{2+}]_i$  in chromaffin cell populations (e.g.

Knight and Kesteven, 1983; Kao and Schneider, 1985; O'Sullivan and Burgoyne, 1989; Stauderman and Pruss, 1989) or single cells (O'Sullivan *et al.* 1989; Cheek *et al.* 1989*a,b*; Kim and Westhead, 1989; Stauderman *et al.* 1990; Ito *et al.* 1991). From these studies, resting  $[Ca^{2+}]_i$  in the chromaffin cell appears to be in the range  $10\text{--}100\text{nmol l}^{-1}$  and, after stimulation, this rises to  $300\text{--}1000\text{nmol l}^{-1}$ . An assessment of the relationship between the  $Ca^{2+}$  response and the triggering of secretion has resulted in the consensus that the major signal for secretion is a rise in  $[Ca^{2+}]_i$  due to  $Ca^{2+}$  entry from the extracellular medium (Cheek *et al.* 1989*a,b*; Bunn and Marley, 1989; Kim and Westhead, 1989; Ballesta *et al.* 1989; Stauderman *et al.* 1990; Yamagami *et al.* 1991). The key observation supporting this being that agents which release internally stored  $Ca^{2+}$ , such as  $\text{Ins}(1,4,5)P_3$ -mobilising hormones or less-specific  $Ca^{2+}$ -mobilising compounds such as caffeine and thapsigargin, all fail to promote secretion in the absence of external  $Ca^{2+}$ . Thus, the efficacy of a given agonist reflects the degree to which it is able to promote  $Ca^{2+}$  entry through either voltage-dependent or voltage-independent pathways rather than its ability to elevate  $[Ca^{2+}]_i$  *per se*. By using video-imaging techniques to visualise stimulus-induced changes in  $[Ca^{2+}]_i$  (Burgoyne, 1991; Cheek, 1991), and whole-cell patch-clamp (Augustine and Neher, 1992*a*; Neher and Augustine, 1992) and flash photolysis techniques (Neher and Zucker, 1993) to manipulate  $[Ca^{2+}]_i$  directly at the single-cell level, considerable light has been shed on why  $Ca^{2+}$  influx is so crucial for secretion: it seems likely that only entry of  $Ca^{2+}$  from the external medium delivers  $Ca^{2+}$  in sufficient amounts to the subplasmalemmal exocytotic sites to activate the fusion process (Fig. 1). The difference between  $Ca^{2+}$  entry and  $Ca^{2+}$  mobilisation, as far as exocytosis is concerned, is due to the differing  $Ca^{2+}$  gradients generated, which can lead to a similar mean  $[Ca^{2+}]_i$  within the cell but to a quite different  $[Ca^{2+}]_i$  at the plasma membrane.

### Voltage-dependent channels and $Ca^{2+}$ entry

Nicotinic or GABA stimulation of chromaffin cells results in membrane depolarisation: nicotine *via*  $Na^+$  influx (Kilpatrick *et al.* 1982) and GABA *via*  $Cl^-$  efflux (Bormann and Clapham, 1985). As a result,  $Ca^{2+}$  enters the cell from the outside medium through voltage-dependent  $Ca^{2+}$  channels, or *via* the acetylcholine receptor itself in the case of nicotine (Holz *et al.* 1982; Kilpatrick *et al.* 1982). Chromaffin cells possess both L-type dihydropyridine-sensitive  $Ca^{2+}$  channels (Artalejo *et al.* 1987; Cena *et al.* 1989) and dihydropyridine-insensitive  $\omega$ -conotoxin-sensitive  $Ca^{2+}$  channels (Rosario *et al.* 1989) as well as other pharmacologically distinct  $Ca^{2+}$  channels (Artalejo *et al.* 1991). L-type channels are not the only channels involved in voltage-dependent  $Ca^{2+}$  entry leading to secretion because dihydropyridine antagonists do not completely block the rise in  $[Ca^{2+}]_i$  or the secretion elicited by either nicotine or GABA (Kataoka *et al.* 1988; Kitayama *et al.* 1990). Electrophysiological studies on single-channel kinetics have shown that chromaffin cells also possess a class of dihydropyridine-sensitive  $Ca^{2+}$  channel that is recruited in response to depolarisation to form a facilitation current whose role in exocytosis has yet to be determined (Artalejo *et al.* 1991).

Spatial aspects of the  $Ca^{2+}$  signal in single bovine chromaffin cells have been determined using video-imaging of Fura-2-loaded intact cells (O'Sullivan *et al.* 1989;

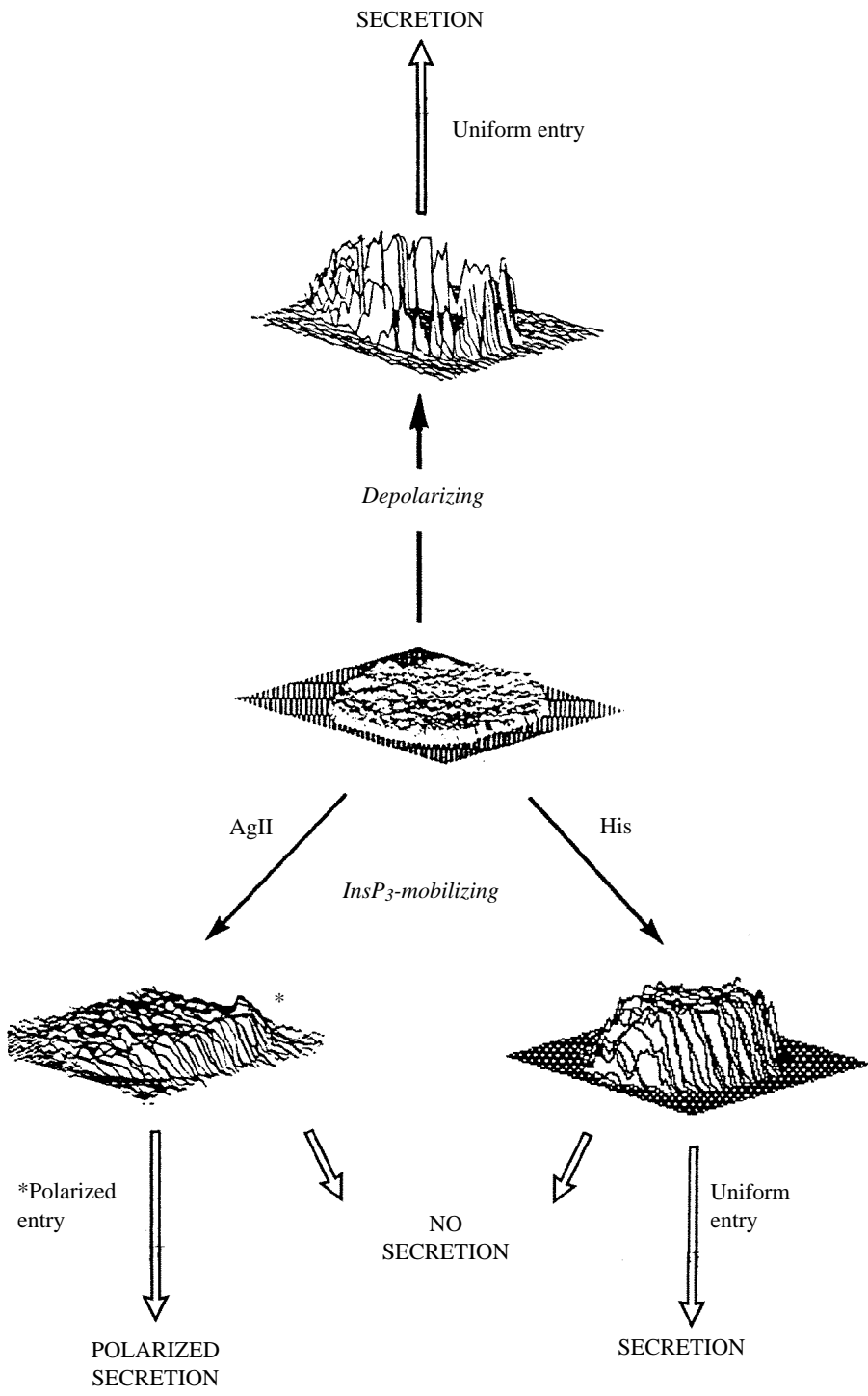


Fig. 1

Cheek *et al.* 1989*a,b*, 1990; Burgoyne *et al.* 1989; Ito *et al.* 1991) and Fura-2 in whole-cell patch-clamp recording (Neher and Augustine, 1992). Following stimulation with nicotine or electrical depolarisation, the rise in  $[Ca^{2+}]_i$  due to  $Ca^{2+}$  influx was initially restricted to the sub-plasmalemmal region of the cell (O'Sullivan *et al.* 1989; Cheek *et al.* 1989*b*; Neher and Augustine, 1992). An increase in  $[Ca^{2+}]_i$  then spread into the interior of the cell to give a more uniform elevation of  $[Ca^{2+}]_i$  throughout the cytoplasm. Subsequently, a further increase in  $[Ca^{2+}]_i$  occurred that was sometimes localised to one area of the cell (O'Sullivan *et al.* 1989). This latter response may be due to the release of  $Ca^{2+}$  from internal stores (see below). The early (<1s) subplasmalemmal rise in  $[Ca^{2+}]_i$  was calculated to be 40–50 nmol l<sup>-1</sup> above the resting level (O'Sullivan *et al.* 1989). The temporal resolution of the imaging system used (5ratioimages s<sup>-1</sup>: see Moreton, 1991) is such that a  $Ca^{2+}$  wave spreading at a rate of about 10  $\mu\text{ms}^{-1}$  has to diffuse about 2  $\mu\text{m}$  from its point source (the channel in the plasma membrane) before it can be detected. As  $Ca^{2+}$  spreads this distance from its point source, its concentration has been predicted to decay exponentially (Fig. 2) from a concentration in the vicinity of the mouth of the  $Ca^{2+}$  channel that is probably in the range 10–100  $\mu\text{mol l}^{-1}$  (Smith and Augustine, 1988). Recent studies on chromaffin cells using whole-cell-attached patches have more directly indicated that these high levels of  $Ca^{2+}$  can exist at the sites of exocytosis in stimulated cells: when raising  $[Ca^{2+}]_i$  by dialysis, even 100  $\mu\text{mol l}^{-1}$   $[Ca^{2+}]_i$  did not achieve the same rates of secretion as those seen in response to depolarisation, as judged by measuring the change in capacitance across the plasma membrane (Augustine and Neher, 1992*a*). Unfortunately, such spatially restricted high levels of  $[Ca^{2+}]_i$  cannot reliably be visualised with current imaging technology, not only because of the limited temporal and spatial resolution of the imaging systems but also because the majority of dyes currently available to monitor  $[Ca^{2+}]_i$  saturate in the range 1–2  $\mu\text{mol l}^{-1}$ .

The voltage-operated  $Ca^{2+}$  channels responsible for delivering this high  $[Ca^{2+}]_i$  to the sites of exocytosis are probably located over the entire cell surface: by visualising the sites of exocytosis using an antibody raised against the chromaffin granule membrane protein dopamine- $\beta$ -hydroxylase, it has been shown that nicotine-induced depolarisation triggers a secretory response that originates from the entire surface of the cell (Cheek *et al.* 1989*a*).

An interesting variation on this theme appears to exist in fast synapses, where  $Ca^{2+}$  entry through voltage-dependent channels is also the key trigger for exocytosis. Various experimental approaches have led to the idea that rapid physiological transmitter release may occur only from synaptic vesicles very close to  $Ca^{2+}$  channels (Augustine *et al.* 1991). Recent work using a low-affinity form of the  $Ca^{2+}$ -sensitive photoprotein aequorin has shown the presence of microdomains of  $Ca^{2+}$  in the squid giant synapse where  $[Ca^{2+}]_i$

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Fig. 1. Summary of intracellular  $Ca^{2+}$  signals generated in bovine adrenal chromaffin cells by various agonists and their relationship to the triggering of exocytosis. The subcellular distribution of  $[Ca^{2+}]_i$  is shown in the form of three-dimensional plots. The most effective stimuli for exocytosis are those that depolarise the cells, allowing uniform  $Ca^{2+}$  entry through voltage-dependent  $Ca^{2+}$  channels (top). Ins(1,4,5) $P_3$ -mobilising agonists release  $Ca^{2+}$  from internal stores, but it is the associated polarised or uniform  $Ca^{2+}$  entry in some cells that leads to secretion. AgII, angiotensin II; His, histamine.

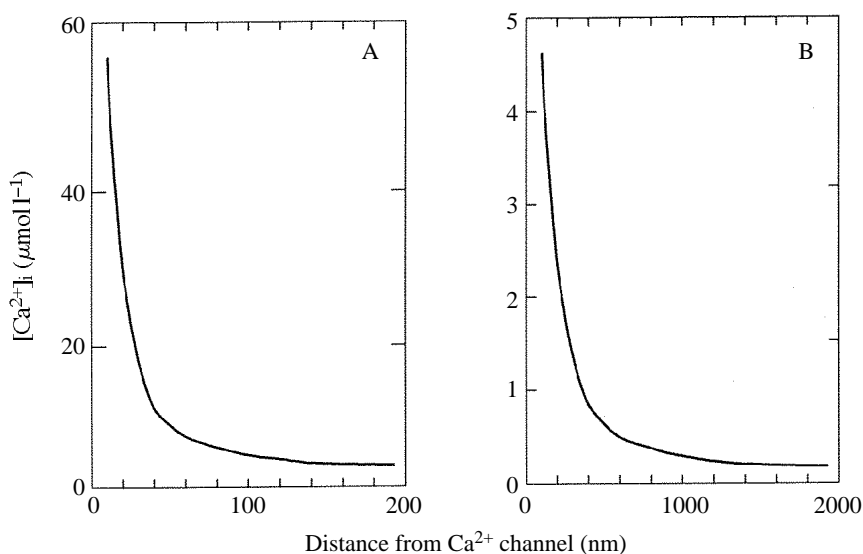


Fig. 2. Theoretical  $[Ca^{2+}]_i$  at varying distances from the plasma membrane 1ms after opening of voltage-dependent  $Ca^{2+}$  channels. (A) Higher  $[Ca^{2+}]_i$  at shorter distances from the plasma membrane and (B) lower  $[Ca^{2+}]_i$  at longer distances. Redrawn from Smith and Augustine (1988).

can reach 200–300  $\mu\text{mol l}^{-1}$  during stimulation (Llinas *et al.* 1992), which may explain why exocytosis can occur within a few hundred microseconds in these very specialised cells. Voltage-dependent  $Ca^{2+}$  entry may also be localised in small secretory cells such as rat pituitary gonadotropes (Rawlings *et al.* 1991), but whether this triggers localised exocytotic secretion is not known.

#### *Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release*

A secondary rise in  $[Ca^{2+}]_i$ , that follows  $Ca^{2+}$  influx, is observed on continued depolarisation of chromaffin cells (O'Sullivan *et al.* 1989), but not after stimulation with a single action potential (Neher and Augustine, 1992). An interesting possibility is that this elevation in  $[Ca^{2+}]_i$  is due to  $Ca^{2+}$  release from internal stores that only become sensitised following the greater  $Ca^{2+}$  influx (Hua *et al.* 1993) resulting from activation of the facilitation  $Ca^{2+}$  current, because this current only occurs with repetitive depolarisation (see above; Artalejo *et al.* 1991).  $Ca^{2+}$  mobilisation could occur either from stores that are sensitive to  $\text{Ins}(1,4,5)P_3$  or by a process of  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR) from stores containing caffeine-sensitive ryanodine receptors. In favour of the first mechanism, it has been shown that both nicotine and high  $[K^+]$  result in the generation of  $\text{Ins}(1,4,5)P_3$  in bovine chromaffin cells due to  $Ca^{2+}$ -dependent activation of phosphoinositidase C (Eberhard and Holz, 1987, 1988). However, a process of CICR similar to that characterised in muscle cells has been proposed to occur in non-muscle cells (Berridge, 1993), and this could be responsible for the secondary rise in  $[Ca^{2+}]_i$ . CICR in muscle cells is mediated by a ryanodine receptor that is caffeine-sensitive.

Chromaffin cells contain caffeine-sensitive  $\text{Ca}^{2+}$  stores (Liu *et al.* 1991) evenly distributed throughout the cytoplasm (Burgoyne *et al.* 1989; Cheek *et al.* 1990) and these are, at least in part,  $\text{Ins}(1,4,5)\text{P}_3$ -insensitive (Robinson and Burgoyne, 1991; Cheek *et al.* 1991a; Stauderman *et al.* 1991). Several lines of evidence indicate that these stores may contribute to the  $\text{Ca}^{2+}$  signal. Emptying caffeine-sensitive stores reduces the magnitude of the histamine-induced rise in  $[\text{Ca}^{2+}]_i$  in bovine chromaffin cells (Stauderman and Murawsky, 1991), and the sensitivity of the store to caffeine has been reported to be regulated by the level of luminal free  $\text{Ca}^{2+}$ , as is the corresponding store in muscle cells (Cheek *et al.* 1991a, 1993a). In rat chromaffin cells, spontaneous fluctuations in  $[\text{Ca}^{2+}]_i$  were generated by an  $\text{Ins}(1,4,5)\text{P}_3$ -insensitive but caffeine-sensitive store (Malgaroli *et al.* 1990). Caffeine is unable to trigger significant secretion from intact bovine chromaffin cells, but the state of filling of the store has been shown to influence the decay time of a depolarisation-induced  $\text{Ca}^{2+}$  signal (Cheek *et al.* 1990). The role of the caffeine-sensitive store in  $\text{Ca}^{2+}$  signalling and bovine chromaffin cell function still has to be fully elucidated but it may be that the store is involved more in  $\text{Ca}^{2+}$  homeostasis than in the direct triggering of exocytosis. Whether the putative messenger molecule cyclic ADP-ribose (Galione, 1993) is able to promote  $\text{Ca}^{2+}$  release from the caffeine-sensitive store in chromaffin cells has yet to be investigated.

### **$\text{Ins}(1,4,5)\text{P}_3$ -mobilising hormones**

Muscarinic agonists raise  $[\text{Ca}^{2+}]_i$  in chromaffin cells by stimulating release from internal stores (Kao and Schneider, 1985; Cheek and Burgoyne, 1985), but this does not promote secretion from bovine cells (Fisher *et al.* 1981; Cheek and Burgoyne, 1985; Ballesta *et al.* 1989; Yamagami *et al.* 1991). Within a population, not all chromaffin cells respond to muscarinic receptor activation and, in those that do, the early subplasmalemmal rise in  $[\text{Ca}^{2+}]_i$  seen in response to depolarisation (Fig. 1) does not occur. Instead, the rise in  $[\text{Ca}^{2+}]_i$  initiates in a more localised region (arrow, Fig. 3C) that appears to co-localise with a particular domain of the endoplasmic reticulum/nuclear envelope organelle (asterisk, Fig. 3A). One possibility is that the local area of initiation of the  $\text{Ca}^{2+}$  signal represents a subdomain of the organelle containing the most sensitive  $\text{Ins}(1,4,5)\text{P}_3$  receptors, as has been suggested in exocrine secretory cells (Thorn *et al.* 1993). It is likely that in intact cells such a local release of  $\text{Ca}^{2+}$  does not result in a sufficient elevation in  $[\text{Ca}^{2+}]_i$  at the exocytotic sites to activate the fusion process, hence the lack of secretory response. This idea is supported by the findings that non-specific release of  $\text{Ca}^{2+}$  from the  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store in response to thapsigargin was also incapable of triggering release, unless there was a secondary influx of external  $\text{Ca}^{2+}$  (Cheek and Thastrup, 1989), and that caffeine, while considerably elevating average  $[\text{Ca}^{2+}]_i$  by releasing internal stores, was also ineffective at triggering secretion (Cheek *et al.* 1990). All these data indicate that the optimal  $\text{Ca}^{2+}$  signal required for secretion is a large ( $>10 \mu\text{mol l}^{-1}$ ) rise in  $[\text{Ca}^{2+}]_i$  immediately beneath the plasma membrane and that, in intact cells, this is most effectively achieved by  $\text{Ca}^{2+}$  entry and not by  $\text{Ca}^{2+}$  release from internal stores. Interestingly, in whole-cell-attached patched cells, elevating  $[\text{Ca}^{2+}]_i$  to  $10 \mu\text{mol l}^{-1}$  by releasing  $\text{Ca}^{2+}$  from intracellular stores has been found to trigger

secretion (Augustine and Neher, 1992). Presumably under experimental conditions in which the  $\text{Ca}^{2+}$ -buffering capacity of the cell is reduced, sufficient  $\text{Ca}^{2+}$  is able to reach the plasma membrane from the internal stores to activate the exocytotic sites.

After initiation in a localised region of the cell (see Fig. 3), the  $\text{Ca}^{2+}$  signal in response to the  $\text{Ins}(1,4,5)\text{P}_3$ -mobilising stimulus histamine has been found to spread across the cell in the form of a wave (Fig. 4), in a manner similar to that reported for other cell types (Berridge, 1993). As mentioned above, pretreatment of cells with caffeine attenuated the histamine-induced  $\text{Ca}^{2+}$  response (Stauderman and Murawsky, 1991), implicating the caffeine-sensitive store in mediating the propagation phase of the signal, perhaps by a process of CICR as already described. It should be noted, however, that caffeine has subsequently been reported to be able to inhibit  $\text{Ins}(1,4,5)\text{P}_3$ -mediated  $\text{Ca}^{2+}$  release in certain tissues (Brown *et al.* 1992), so whether the  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store or the caffeine-sensitive store is responsible for propagating the  $\text{Ca}^{2+}$  wave in chromaffin cells remains to be clarified.

#### *$\text{Ca}^{2+}$ entry in response in $\text{Ins}(1,4,5)\text{P}_3$ -mobilising hormones*

Histamine and angiotensin II both trigger secretion that depends upon external  $\text{Ca}^{2+}$  (Noble *et al.* 1988; O'Sullivan *et al.* 1989; Stauderman *et al.* 1990), with histamine being much more potent than angiotensin II. This may be because the angiotensin receptor, but not the histamine receptor, desensitises rapidly, thereby shutting off  $\text{Ca}^{2+}$  entry (Stauderman *et al.* 1990). Additionally, the localisation of  $\text{Ca}^{2+}$  entry in response to the two stimuli may differ. Angiotensin II causes an initial local rise in  $[\text{Ca}^{2+}]_i$  and a polarised entry of external  $\text{Ca}^{2+}$ , as judged by visualising the quenching of Fura-2 fluorescence by the  $\text{Ca}^{2+}$  surrogate  $\text{Mn}^{2+}$  (Cheek *et al.* 1989a, 1993b). This results in a low level of catecholamine release (O'Sullivan and Burgoyne, 1989; Bunn and Marley, 1989) that is also polarised (Cheek *et al.* 1989a). The more potent secretagogue histamine, at concentrations optimal for secretion, does not open voltage-dependent  $\text{Ca}^{2+}$  channels (Artalejo *et al.* 1993), but nevertheless stimulates  $\text{Ca}^{2+}$  entry around the whole plasma membrane, explaining its efficacy in stimulating secretion (Cheek *et al.* 1993b).

The nature of the channels through which angiotensin II and histamine stimulate  $\text{Ca}^{2+}$  entry and the mechanism of their opening are unknown.  $\text{Mn}^{2+}$  entry has been reported to be activated by  $\text{Ca}^{2+}$ -ATPase inhibitors such as thapsigargin in the absence of surface

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Fig. 3. The initiation site of the  $\text{Ca}^{2+}$  signal induced by an  $\text{Ins}(1,4,5)\text{P}_3$ -mobilising muscarinic stimulus in a chromaffin cell and its co-localisation with the endoplasmic reticulum/nuclear envelope organelle. (A) The cell was stained with an antibody to the major endoplasmic reticulum glycoprotein, endoplasmic reticulum chaperone, which revealed the nuclear envelope (N) in addition to the endoplasmic reticulum (asterisk). (B,C,D) The distribution of  $[\text{Ca}^{2+}]_i$  at rest (B), and at 5s (C) and 10s (D) after stimulation with methacholine. The elevation in  $[\text{Ca}^{2+}]_i$  (yellow) is clearly initiated in a central region of the cell, remote from the subplasmalemmal region (arrow, C).

Fig. 4. The initiation site (arrow) of the  $\text{Ca}^{2+}$  signal induced by an  $\text{Ins}(1,4,5)\text{P}_3$ -mobilising histaminergic stimulus in a chromaffin cell and its subsequent propagation across the cell in the form of a wave. The cell was stimulated with  $10\ \mu\text{mol l}^{-1}$  histamine in an EGTA-containing extracellular medium at time zero. The location of the nucleus (n) was revealed by ethidium bromide staining after video-imaging of Fura-2.



receptor stimulation (Robinson *et al.* 1992), indicating the existence of a capacitative entry mechanism in these cells (Putney, 1990). Whether such a mechanism is responsible for all of the entry observed after receptor activation, however, remains to be elucidated.  $\text{Ca}^{2+}$  entry in response to angiotensin II was observed after prior depletion of the internal  $\text{Ca}^{2+}$  store with ionomycin, suggesting that, even with depleted stores, entry could still be stimulated by receptor activation (Stauderman and Pruss, 1989). One possibility is that hormones directly open a receptor-operated channel (ROC) in the plasma membrane, as does ADP in platelets (Sage *et al.* 1989) and ATP in PC12 cells (V. A. Barry and T. R. Cheek, in preparation). An alternative possibility is that an intracellular messenger, such as  $\text{Ins}(1,4,5)\text{P}_3$  (Kuno and Gardner, 1987) or inositol 1,4,5-tetrakisphosphate [ $\text{Ins}(1,3,4,5)\text{P}_4$ ] (Irvine, 1992), may promote  $\text{Ca}^{2+}$  entry through a second-messenger-operated channel. Angiotensin II and histamine both result in a transient generation of  $\text{Ins}(1,4,5)\text{P}_3$  within 10s in chromaffin cells. The response to histamine, but not to angiotensin II, was biphasic with a secondary generation of  $\text{Ins}(1,4,5)\text{P}_3$  occurring at 20s (Stauderman and Pruss, 1990). The secondary increase in  $\text{Ins}(1,4,5)\text{P}_3$  in response to histamine was paralleled by an increase in  $\text{Ins}(1,3,4)\text{P}_3$  that was absent after stimulation with angiotensin II. This  $\text{Ins}(1,3,4)\text{P}_3$  could only have originated from dephosphorylation of  $\text{Ins}(1,3,4,5)\text{P}_4$ , suggesting that histamine produces more  $\text{Ins}(1,3,4,5)\text{P}_4$ , as well as more  $\text{Ins}(1,4,5)\text{P}_3$ , than does angiotensin II. With respect to eliciting a secretory response, it may be significant that histamine is more potent than angiotensin II in generating both  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ins}(1,3,4,5)\text{P}_4$  because both of these inositol phosphates have been proposed to trigger  $\text{Ca}^{2+}$  entry into cells (see Irvine, 1992). There is as yet no direct evidence supporting a role for  $\text{Ins}(1,3,4,5)\text{P}_4$  in gating  $\text{Ca}^{2+}$  entry into chromaffin cells, but Mochizuki-Oda *et al.* (1991) have demonstrated that  $\text{Ins}(1,4,5)\text{P}_3$  is capable of directly opening a plasma membrane channel that gates  $\text{Ca}^{2+}$  in these cells. With reference to this last point, a recent study has indicated that chromaffin granules may be  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive (Yoo and Albanesi, 1990). Exocytosis of these granules, with the resulting incorporation of the  $\text{Ins}(1,4,5)\text{P}_3$  receptor into the cytosolic surface of the plasma membrane, may be a means of targeting the  $\text{Ins}(1,4,5)\text{P}_3$  receptor to the plasma membrane.

Other possible intracellular messenger activators of plasma membrane  $\text{Ca}^{2+}$  channels, yet to be explored in chromaffin cells, include  $\text{Ca}^{2+}$  itself (von Tschärner *et al.* 1986) and GTP (Mullaney *et al.* 1988).

### **$\text{Ca}^{2+}$ signals and secretion in PC12 cells**

A remarkable demonstration of the physiological importance of targeting the elevation in  $[\text{Ca}^{2+}]_i$  to the strategic area of the cell responsible for exocytosis is shown in Fig. 5.

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Fig. 5. Differential effects of extracellular ATP on  $\text{Ca}^{2+}$  waves and secretion in PC12 cells. In the absence of extracellular  $\text{Ca}^{2+}$ ,  $100 \mu\text{mol l}^{-1}$  ATP releases  $\text{Ca}^{2+}$  from intracellular stores, triggering a wave of  $\text{Ca}^{2+}$  in some cells (A), but this  $\text{Ca}^{2+}$  signal is unable to activate exocytosis (C). In the presence of extracellular  $\text{Ca}^{2+}$ , ATP promotes only  $\text{Ca}^{2+}$  entry in some cells (B), and this results in a sufficient elevation in subplasmalemmal  $[\text{Ca}^{2+}]_i$  to trigger exocytosis (C).

Video-imaging experiments using PC12 cells have revealed that, in the absence of extracellular  $\text{Ca}^{2+}$ , approximately 40% of single cells responded to extracellular ATP with a transient elevation in  $[\text{Ca}^{2+}]_i$  and that sometimes this was in the form of an initially localised  $\text{Ca}^{2+}$  signal propagated across the cell in the form of a wave (Fig. 5A). Conversely, in the presence of extracellular  $\text{Ca}^{2+}$ , more than 90% of single cells responded with a transient elevation in  $[\text{Ca}^{2+}]_i$  to an application of ATP, with  $\text{Ca}^{2+}$  often initially localised to the subplasmalemmal region before infilling into the cell (Fig. 5B). Further experiments using the  $\text{Ca}^{2+}$  surrogate  $\text{Mn}^{2+}$ , and the nucleotide UTP to desensitise the release of intracellularly stored  $\text{Ca}^{2+}$ , have indicated that this subplasmalemmal elevation in  $[\text{Ca}^{2+}]_i$  is due to ATP triggering  $\text{Ca}^{2+}$  entry independently of intracellular  $\text{Ca}^{2+}$  mobilisation (V. A. Barry and T. R. Cheek, in preparation), presumably through a putative receptor-operated channel (Sela *et al.* 1991).

Interestingly,  $[\text{^3H}]$ dopamine secretion from populations of PC12 cells was only triggered by ATP in the presence of external  $\text{Ca}^{2+}$  (Fig. 5C), indicating that a subplasmalemmal elevation in  $[\text{Ca}^{2+}]_i$ , and not a release of intracellularly stored  $\text{Ca}^{2+}$  in the form of a wave, is most effective at activating exocytotic fusion. These experiments indicate that the same stimulus can deliver  $\text{Ca}^{2+}$  signals with different temporal and spatial profiles into the same cell type by activating different signalling pathways and, most importantly, that these  $\text{Ca}^{2+}$  signals can be functionally distinct.

### Direct manipulation of $[\text{Ca}^{2+}]_i$

The main conclusion to emerge from studies in which stimulus-induced changes in  $[\text{Ca}^{2+}]_i$  in chromaffin cells have been monitored at the single-cell level is that  $\text{Ca}^{2+}$  entry is essential for the activation of exocytosis, and this is likely to be because only entry delivers  $\text{Ca}^{2+}$  in sufficient amounts to the subplasmalemmal exocytotic sites (Cheek *et al.* 1989*a,b*, 1991*b*, 1993*b*; Neher and Augustine, 1992; Augustine and Neher, 1992*b*; Neher and Zucker, 1993).

As already discussed, because of technical limitations, the  $[\text{Ca}^{2+}]_i$  existing within a few nanometres of exocytotic sites cannot yet be directly visualised (see also Augustine and Neher, 1992*b*), but both theoretical calculations (Smith and Augustine, 1988) and patch-clamp experiments (Neher and Augustine, 1992; Augustine and Neher, 1992*a*) indicate that such intracellular  $\text{Ca}^{2+}$  concentrations probably lies in the range  $10\text{--}100\ \mu\text{mol l}^{-1}$  before dissipating very rapidly (approximately  $100\text{--}200\text{ms}$ ) into the cytosol. Using the technique of flash photolysis and caged  $\text{Ca}^{2+}$ , Neher and Zucker (1993) have elevated average  $[\text{Ca}^{2+}]_i$  in chromaffin cells to these predicted levels while simultaneously monitoring secretion by measuring plasma membrane capacitance. Elevating  $[\text{Ca}^{2+}]_i$  to above  $10\ \mu\text{mol l}^{-1}$  was found to trigger ultrafast ( $<50\text{ms}$ ) exocytosis, possibly resulting from the release of granules already docked at the plasma membrane, while elevating  $[\text{Ca}^{2+}]_i$  to the range  $70\text{--}150\ \mu\text{mol l}^{-1}$  was found, in addition, to trigger both a fast ( $1\text{--}2\text{s}$ ) and a slow ( $10\text{--}30\text{s}$ ) exocytotic response, perhaps representing the release of granules restrained within the subplasmalemmal cytoskeleton or recruited from deep within the cell. Similar findings have been made for pituitary melanotrophs (Thomas *et al.* 1993).

Clearly, experiments such as these, in which  $[Ca^{2+}]_i$  can be changed rapidly and uniformly in a known manner, will be central to unravelling the multiplicity of  $Ca^{2+}$ -dependent processes that are related to the secretory response in chromaffin and other excitable cell types.

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