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# CONTROL OF SECRETION IN ANTERIOR PITUITARY CELLS – LINKING ION CHANNELS, MESSENGERS AND EXOCYTOSIS

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## Summary

Normal anterior pituitary cells, in their diversity and heterogeneity, provide a rich source of models for secretory function. However, until recently they have largely been neglected in favour of neoplastic, clonal tumour cell lines of pituitary origin, which have enabled a number of studies on supposedly homogeneous cell types. Because many of these lines appear to lack key peptide and neurotransmitter receptors, as well as being degranulated with accompanying abnormal levels of secretion, we have developed a range of normal primary anterior pituitary cell cultures using dispersion and enrichment techniques. By studying lactotrophs, somatotrophs and gonadotrophs we have revealed a number of possible transduction mechanisms by which receptors for hypothalamic peptides and neurotransmitters may control secretion. In particular, the transduction events controlling secretion from pituitary cells may differ fundamentally from those found in other cell types.

Patch-clamp recordings in these various pituitary cell preparations have revealed substantial populations of voltage-dependent $Na^+$, $Ca^{2+}$ and $K^+$ channels which may support action potentials in these cells. Although activation of these channels may gate $Ca^{2+}$ entry to the cells under some conditions, our evidence taken with that of other laboratories suggests that peptide–receptor interactions leading to hormone secretion occur independently of significant membrane depolarization. Rather, secretion of hormone and rises in intracellular calcium measured with new probes for intracellular calcium activity, can occur in response to hypothalamic peptide activation in the absence of substantial changes in membrane potential. These changes in intracellular calcium activity almost certainly depend on both intracellular and extracellular calcium sources. In addition, strong evidence of a role for multiple intracellular receptors and modulators in the secretory event suggests we should consider the plasma

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membrane channels important for regulation of hormone secretion to be predominantly agonist-activated, rather than of the more conventional voltage-dependent type. Likewise, evidence from new methods for recording single ion channels suggests the existence of intracellular sites for channel modulation, implying they too may play an important role in secretory regulation.

We shall consider new data and new technology which we hope will provide key answers to the many intriguing questions surrounding the control of pituitary hormone secretion. We shall highlight our work with recordings of single ion channels activated by peptides, and recent experiments using imaging of intracellular ionized free calcium. In addition, we shall discuss promising new results combining several novel methodologies which are enabling recordings of the electrical manifestation of secretory granule fusion, using measurement of extremely small changes in membrane capacitance. These studies may provide an interesting way forward in permitting examination of the time course of both granule fusion and resulting membrane recovery. In summary, if any maxim has been proved, it is that the more we know, the less we understand!

## Introduction

Cells of the anterior pituitary gland are the source of important physiological effector hormones. This endocrine tissue secretes growth hormone (GH), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), beta-lipotropin, beta-endorphin and melanocyte-stimulating hormone (MSH). This wide spectrum of hormones in turn controls such important bodily functions as growth, reproduction and fertility, milk production, thyroid hormone output, and maternal behaviour. In most cases, the 'one cell – one hormone' concept seems to apply.

It is clear that most pituitary cells have receptors for small-peptide-releasing factors which are synthesized by and secreted from discrete groups of cells in the hypothalamus, ultimately finding their way to the anterior pituitary gland through a specialized portal circulation. Many of these cells also possess receptors for neurotransmitter substances such as $\gamma$-aminobutyric acid (GABA), acetylcholine, noradrenaline and dopamine, which appear to modulate hormone secretion. Given the heterogeneity of the anterior pituitary gland, how should one set about studying the secretion of a particular hormone from a particular cell type?

One approach was provided by Tashjian's group working, not on normal cells, but on clonal cells derived from a rat pituitary cell tumour (Tashjian *et al.* 1968). This work produced the most widely studied group of pituitary tumour cell lines available, the GH3 and GH4 clonal lines and their various sub-clones. Work on these has provided much detail about basic aspects of endocrine cells but they are poor models for studying specific pituitary cell types. Compared with normal anterior pituitary cells secreting *either* prolactin *or* growth hormone, most GH3/GH4 lines secrete *both* hormones and at abnormally high rates; the cells are virtually totally degranulated; they lack receptors for dopamine (the tonic

inhibitory transmitter implicated in prolactin secretion) and GRF (growth-hormone-releasing factor – the peptide promoting growth hormone secretion from somatotrophs). There was clearly a strong case for wishing to study the properties of single, identified cells associated with secretion of a particular hormone.

Most groups, including our own, have relied on separation and enrichment of cell populations on the basis of cell density. For prolactin and growth hormone cells, we have used the density centrifugation technique employing isotonic Percoll gradients (Mason & Ingram, 1986). Another approach has been to identify the cells using immunological techniques such as the reverse haemolytic plaque assay (DeRiemer & Sakmann, 1986; Lingle, Sombati & Freeman, 1986), which can detect hormone secretion from a single cell, or by specific fluorescence labelling of cells (Marchetti, Childs & Brown, 1987). More recently, anterior pituitary cells have also been separated using fluorescence-activated cell sorting (FACS) with antibodies or cholera toxin as a probe (Lewis, Goodman, St. John & Barker, 1988). In the case of the gonadotrophin-secreting cells, we have employed a different approach altogether, taking advantage of the observation that the ovine pars tuberalis contains gonadotrophs as the only hormone-secreting cell type (Mason & Waring, 1985). However, our approaches provide enrichment, not purification, and we have further employed post-recording staining with highly specific antibodies to ensure certainty of identification for most single-cell work.

## Aims and objectives

Our interest lies broadly in the mechanisms that link the stimulus of a peptide or a neurotransmitter to eventual secretion of hormone from anterior pituitary cells. The aim of this article is to explore the current knowledge about this complex pathway, and how it might be modulated within the cell. At present, we believe this to involve a sequence which may be approximated by: (1) binding of a peptide or a neurotransmitter (a ligand) to its specific plasma membrane receptor on the cell; (2) activation of either ligand–ion channel complexes or intracellular metabolic cascades, the latter producing new sets of molecules (intracellular messengers); (3) interaction of these molecules with membrane ionic channels to cause currents to flow across the plasma membrane and possibly also mobilize ionic current flow within the cell; (4) release of hormone when secretory granules fuse with the plasma membrane in an exocytotic event.

Within this framework, the specific questions we would like to address in this article concern some of the fundamental properties of membrane events responsible for stimulus–secretion coupling. Is hormone secretion a voltage-dependent process, or is it linked to membrane potential in any way? Are electrical events, such as subthreshold fluctuations or action potential firing, important for hormone secretion? How is electrical activity modulated by peptide-releasing factors? Can evidence be found for activation of intracellular messengers that may influence ionic current flow? How are these changes linked ultimately to hormone secretion? Is $Ca^{2+}$ vital for hormone secretion from these cells, or might there be

other substances essential for exocytosis? Hence do changes in intracellular $Ca^{2+}$ concentration occur when cells are stimulated with specific releasing factors? What is the source of changes in cell calcium concentration? Do all types of cell behave in the same way?

## Ion channels in anterior pituitary cells

Ion channels are thought to be integral protein units within the lipid bilayer of a membrane which can in simple terms be regarded as a gate that is either open or closed. The mechanism by which the channel gate is opened or closed depends upon the type of channel. For convenience the channels that have been found in anterior pituitary cells can be classified into a number of different subtypes. These are listed below, and discussed in more detail later in the text.

*Voltage-activated channels*: channels that are activated by a change in membrane potential; e.g. the tetrodotoxin TTX-sensitive $Na^+$ channels and TTX-insensitive $Ca^{2+}$ and $K^+$ channels seen in many anterior pituitary cells.

*Primary ligand-activated channels*: channels that are *directly* coupled to a receptor; e.g. the GABA-activated $Cl^-$ channel seen in bovine lactotrophs (Inenaga & Mason, 1987) and melanotrophs (Kehl, Hughes & McBurney, 1987; Taleb *et al.* 1987).

*Secondary ligand-activated channels*: channels that are gated by a reaction with a molecule whose availability depends on another cellular event such as receptor occupancy or changes in the cellular metabolism; e.g. the gonadotrophin-releasing hormone (GnRH)-activated $Ca^{2+}$ channel in gonadotrophs (Mason & Waring, 1986). Meldolesi & Pozzan (1987) have referred to these as second-messenger-operated calcium channels (SMOCs).

*Voltage-sensitive, intracellular messenger-mediated channels*: channels that are activated by changes in membrane potential, but are also modified by chemical messengers; e.g. the calcium-dependent $K^+$ channel which is opened by calcium, and closed by cyclic AMP.

## Voltage-activated ion channels

Work from a number of laboratories has recently shown that anterior pituitary cells contain a variety of voltage-activated ion channels, many bearing similarities to those found in neurones. Kidokoro (1975) showed that GH3 cells exhibited spontaneous action potentials, and that these were generated partially by $Ca^{2+}$ flux through voltage-activated channels. Thyrotrophin-releasing hormone (TRH), at concentrations known to stimulate PRL release, increased the firing rate in these cells, thus allowing (by implication) more $Ca^{2+}$ into the cell. These data suggest an analogous situation to that found in neurones where firing frequency governs secretion of neurotransmitter. This therefore represented a possible mechanism for the control of hormone release. Work with *normal* anterior pituitary cells has shown that they differ to some extent from GH3 cells, and this work will be discussed below.

Using tight-seal, whole-cell recording techniques, normal anterior pituitary cells have been shown to have resting membrane potentials more negative than −60 mV and a high input resistance (usually 2–20 GΩ). Normal lactotrophs fire action potentials spontaneously, but at a frequency far lower than that seen in neurones (Taraskevich & Douglas, 1979; Cobbett, Ingram & Mason, 1987*a*), usually about 2–3 spikes $s^{-1}$. In both somatotrophs (Mason & Rawlings, 1988) and gonadotrophs (Mason & Sikdar, 1988), we in common with others (Israel, Denef & Vincent, 1986) have not observed any significant level of spontaneous action potentials, although low-frequency, current-evoked action potentials may be observed (Fig. 1B). In all pituitary cells examined, this is because the $Na^+$ currents (see below) require 1–2 s to recover from inactivation following activation. In neoplastic (GH3) rat pituitary cells, action potential firing is also low-frequency – for similar reasons – and has a significant $Ca^{2+}$ component as well as being unaffected by the voltage-activated sodium channel blocker tetrodotoxin (TTX). Normal pituitary cells fire action potentials which are TTX-sensitive, but which cannot be supported by $Ca^{2+}$ alone (Cobbett *et al.* 1987*a*; Mason & Sikdar, 1988). Ironically, despite the presence of these action potentials, release of GH, LH and PRL – either basal or evoked by releasing factors – is not affected by TTX, suggesting the $Na^+$ action potentials may not be important.

Voltage-clamp studies show that anterior pituitary cells possess voltage-activated $Na^+$, $Ca^{2+}$ and $K^+$ channels, and that the currents carried by these channels underlie the action potential. In lactotrophs, for example, a rapidly activating and inactivating, TTX-sensitive $Na^+$ current can be reliably evoked, similar in some respects to that recorded in neurones (Fig. 2A). A much slower inactivating, TTX-insensitive inward current can also be recorded when the external medium contains $Ca^{2+}$ or $Ba^{2+}$ (Fig. 2B). Both inward currents are maximal at about −10 to 0 mV (Fig. 2C).

Under current-clamp, spontaneous depolarizing and hyperpolarizing potential fluctuations of 2–20 mV are often recorded. Since these cells have no synaptic input *in vitro* or *in vivo*, these are proposed to be due to brief, random, spontaneous openings of single ion channels. Because of the cell's high input resistance, spontaneous openings of single ion channels can produce large changes in membrane potential, and depolarizing fluctuations could activate voltage-activated $Ca^{2+}$ channels. Indeed depolarizing voltage fluctuations could be the result of spontaneous opening of voltage-activated $Ca^{2+}$ channels themselves, or some other channel. For instance, a single channel inward current of 1 pA ($10^{-12}$ A) flowing across a resistance typical of whole-cell recorded pituitary cells, say 10 GΩ, would result in a depolarizing voltage of about 10 mV.

In anterior pituitary cells there are two voltage-activated $Ca^{2+}$ channels which may be differentiated by their kinetic properties, and voltage sensitivities (Fig. 3) (DeRiemer & Sakmann, 1986; Lingle *et al.* 1986; Marchetti *et al.* 1987; Cobbett, Ingram & Mason, 1987*b*; Kehl, 1987). One has a low threshold (usually about −30 mV), activates and inactivates rapidly, and can only be reactivated by returning the membrane potential to the original holding potential (−60 mV or

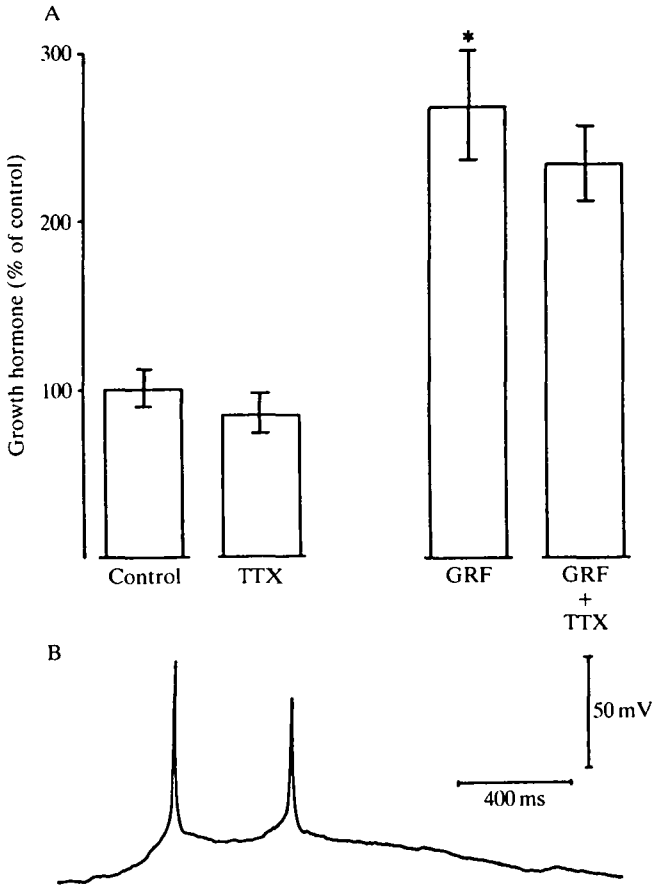



Fig. 1. Spontaneous activity in normal cultured bovine somatotrophs, and the effects of tetrodotoxin (TTX) and growth-hormone-releasing factor (GRF) on growth hormone (GH) secretion from these cells. (A) The percentage of GH released from normal somatotrophs incubated in physiological medium containing the drugs shown for 2h at 37°C. GRF (1 nmol $l^{-1}$) significantly (* = $P < 0{\cdot}01$; Mann–Witney U-test) stimulated GH release from these cells. TTX (10 $\mu$mol $l^{-1}$) had no effect on basal or GRF-stimulated GH release. (B) Spontaneous action potentials recorded from cultured bovine somatotrophs using the whole-cell, patch-clamp technique described in the text. Such action potential generation could be blocked by TTX (not shown).

more negative). The other is activated by voltage pulses to more positive potentials (above −10 mV) and is mainly non-inactivating. The ion channels carrying these currents could be opened during an action potential and thus could mediate $Ca^{2+}$ influx required to support secretion. Because the low-threshold channels are probably substantially inactivated at the resting potential (Cobbett *et al.* 1987*b*), it may be that only the higher-threshold current could contribute to the action potential.

The effects of peptide action and of channel blockers in lactotrophs appear to

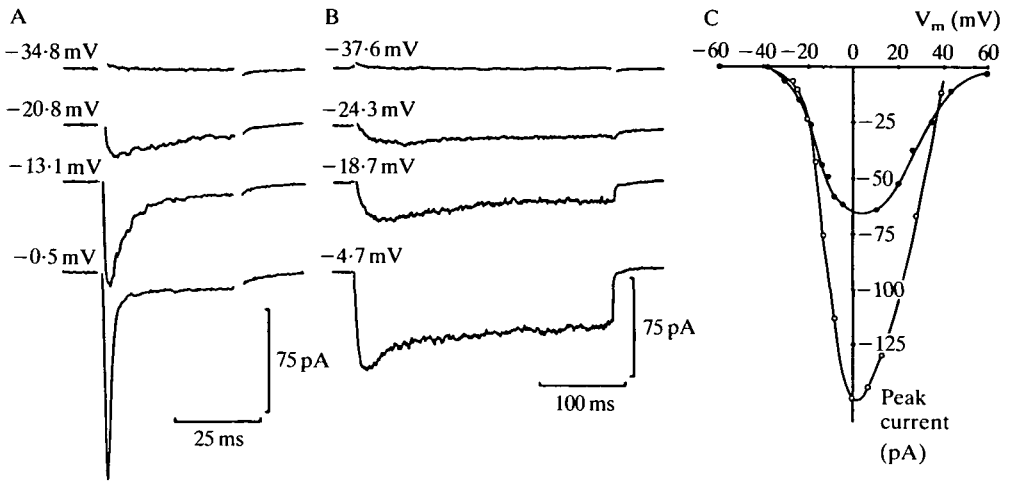



Fig. 2. Voltage-activated inward cation currents in bovine lactotrophs obtained in whole-cell, voltage-clamp recordings. The internal medium contained $Cs^+$ to abolish outward $K^+$ currents. (A) In the presence of external $Na^+$, a rapid transient current was activated by suprathreshold voltage steps from a holding potential of −60 mV. (B) After abolition of the $Na^+$ current by tetrodotoxin and the addition of $Ba^{2+}$ (10 mmol $l^{-1}$) positive voltage steps produced inward currents through $Ca^{2+}$ channels which had a different time course from the $Na^+$ current. (C) The current–voltage relationship of the $Na^+$ (open circles) and $Ba^{2+}$ (closed circles) currents shown in A and B. (Reprinted from Cobbett, Ingram & Mason, 1987*b*, with permission.)

bear some correlation with membrane potential. Application of $Mn^{2+}$ or $Co^{2+}$ to single lactotrophs recorded intracellularly hyperpolarizes the membrane potential (Fig. 4A,B), and TRH application leads to a complex chain of events: initial hyperpolarization followed by a small to negligible depolarization (2–5 mV) and low-frequency firing which is also blocked by $Co^{2+}$ (Fig. 4B). Dopamine, the tonic inhibitory transmitter involved in PRL secretion, strongly depolarizes the membrane potential (Fig. 4C) and suppresses PRL release. Unfortunately, $Co^{2+}$, $Mn^{2+}$ and most divalent cation channel blockers also suppress voltage-activated $Na^+$ currents, as they do in other tissues. Nevertheless, modulation of membrane potential is clearly correlated with hormone secretion.

In GH3 cells, at least, recent work with calcium-sensitive dyes has confirmed that intracellular $Ca^{2+}$ concentration may rise during an evoked action potential or an evoked train of action potentials. But given the failure of a number of groups to observe action potentials in normal cell types, other than lactotrophs, it must be questioned whether the action potential is a ubiquitous mechanism for rises in intracellular calcium level. This point will be discussed in more detail below. In particular, can release be stimulated by a mechanism independent of action potentials, such as subthreshold channel openings and fluctuations in membrane potential close to the resting state?

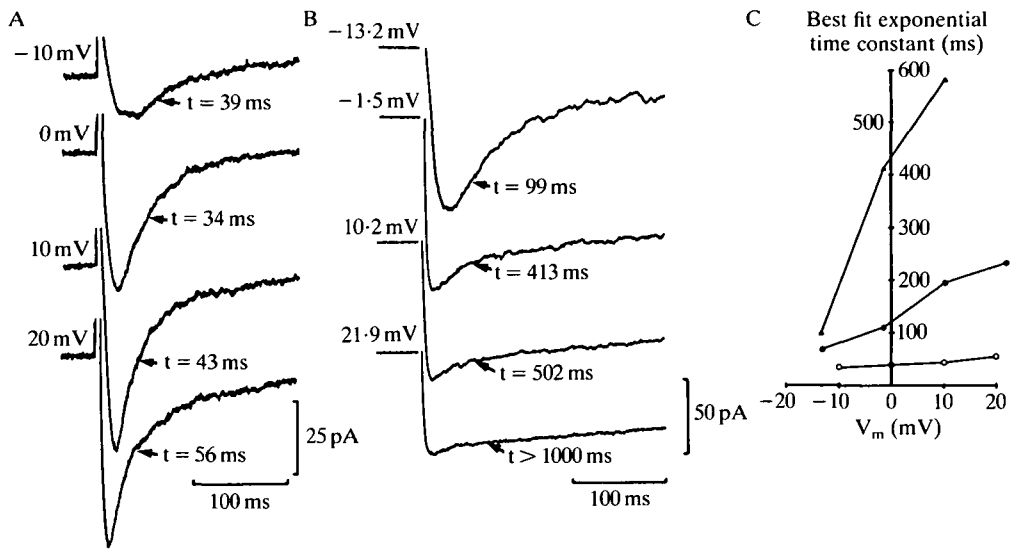



Fig. 3. Voltage-dependence of the time course of inactivation of voltage-activated $Ba^{2+}$ currents recorded from normal bovine lactotrophs. Currents were recorded during positive voltage steps from the holding potential (−70 mV). In some cells the time course of inactivation was relatively independent of the membrane potential (A) whereas in other cells the current seemed to inactivate less at more positive potentials than at more negative test potentials (B). Relationship of the time constant of inactivation and the membrane potential in three representative experiments in which the time constant was highly voltage-dependent (triangles) or relatively voltage-independent (circles). (Reprinted from Cobbett, Ingram & Mason, 1987*b*, with permission.)

## Release-modulating factors and ion channel regulation

The evidence for the involvement of hypophysiotrophic-peptide-activated plasma membrane ion channels comes from different studies. Altering membrane ionic flux either by using classical ion channel blockers – such as those acting upon the $Ca^{2+}$ channel – or by changing the composition of the external medium can alter the hormone release response to releasing factors (Vale, Burgus & Guillemin, 1967 ). Also, the use of electrophysiological techniques has enabled us to study directly the ionic flow through individual ion channels, and these techniques have given irrefutable evidence that the binding of a number of releasing (or release-inhibiting) factors can change channel activity (Mason & Waring, 1985). Whether the altered state of these channels, and the subsequent changes in ionic flux, are critical for secretion is still not fully resolved.

An important role for extracellular $Ca^{2+}$ in the regulation of anterior pituitary hormone secretion was first proposed by Vale and colleagues (Vale *et al.* 1967). They showed that the action of TRH on TSH release from the rat anterior pituitary was dependent upon extracellular calcium, and that high extracellular $K^+$ concentration enhanced TSH release in the presence of calcium. Depolarizing

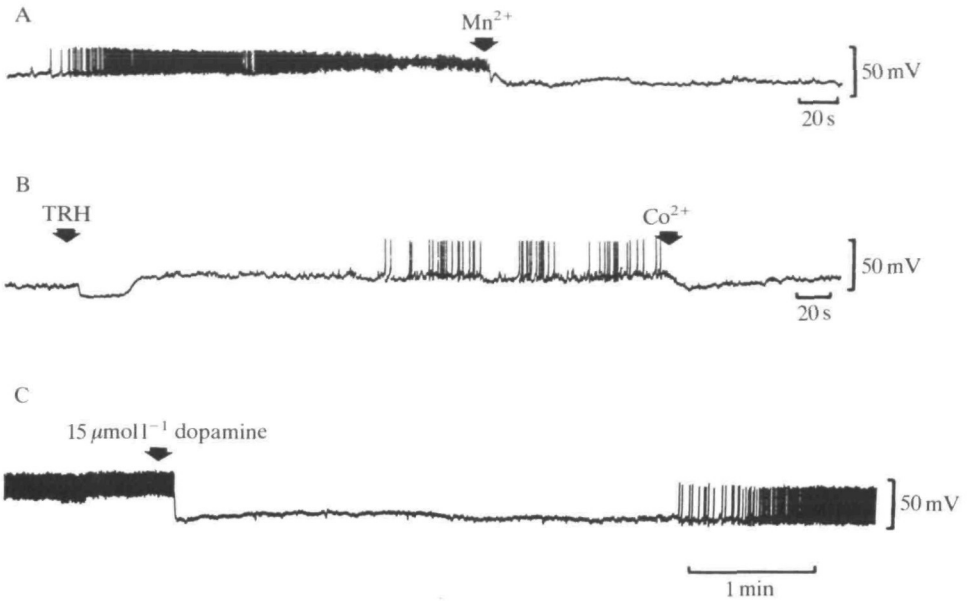



Fig. 4. Conventional intracellular recording from normal bovine lactotroph in culture. (A) Action potentials recorded from a lactotroph in the absence of thyrotrophin-releasing hormone (TRH). Addition of 2 mmol l$^{-1}$ $Mn^{2+}$ hyperpolarized the membrane and blocked action potential firing. (B) Application of 80 nmol l$^{-1}$ TRH triggered a complex chain of events. Initially, the lactotroph hyperpolarized and this was followed by a small depolarization of 2–5 mV, an increase in membrane voltage noise and eventually action potentials which could be blocked by cobalt ions. The initial hyperpolarization is probably caused by activation of a $Ca^{2+}$-activated $K^+$ channel triggered by a rise in intracellular $Ca^{2+}$ concentration. (C) A spontaneously firing lactotroph was markedly inhibited by the application of a bolus of 15 μmol l$^{-1}$ dopamine. As the dopamine was washed away by the perfusion system, the membrane repolarized and action potentials were again observed.

concentrations of $K^+$ were later shown to stimulate hormone release from other anterior pituitary cells (Kraicer *et al.* 1969; MacLeod & Fontham, 1970; Parsons, 1970; Wakabayashi, Kamberi & McCann, 1969). These findings suggested that voltage-dependent influx of $Ca^{2+}$ is one method of linking stimulus to secretion in anterior pituitary cells. Recently Schlegel and colleagues have shown a clear rise in $[Ca^{2+}]_i$ following action potential firing in GH3/B6 cells (Schlegel *et al.* 1987). A 10 ms action potential raised the intracellular calcium to a level sufficient to stimulate hormone secretion. However, there is some evidence to suggest that in other cell types certain factors may cause hormone release in the absence of changes in membrane potential.

GRF is a potent stimulator of GH release from somatotrophs (Guillemin *et al.* 1982). However, intracellular recording has shown that although GRF applied to somatotrophs causes an increase in membrane voltage noise, no absolute change in membrane potential is observed. This noise is blocked by 4 mmol l$^{-1}$ $Co^{2+}$

(Mason *et al.* 1986) suggesting that it is caused directly or indirectly by an increase in the membrane $Ca^{2+}$ permeability. A similar action is seen when ovine gonadotrophs respond to GnRH (Mason & Waring, 1985). Release studies on bovine and ovine cells have shown that GRF-, TRH- and GnRH-stimulated release of GH, PRL and LH, respectively, persists in the presence of doses of TTX capable of blocking action potential firing in these cells (see Fig. 1A) (Mason & Rawlings, 1988; Cobbett *et al.* 1987*a*; Mason & Sikdar, 1988). In these situations, therefore, $Na^+$ action potential firing is not necessary for hormone release caused by releasing factors, and because single, distinct $Ca^{2+}$ action potentials are not observed in the presence of TTX, it must be held in doubt whether action potentials play a role in hormone secretion.

### Receptor- and/or agonist-operated channels

What is the evidence for receptor-operated ion channels? We should consider two different types of agonist effect on channels: primary- and secondary-ligand-activated channels. Meldolesi & Pozzan (1987) have suggested that these should be referred to as receptor-operated (ROC) and second-messenger-operated channels (SMOC), respectively. Evidence for direct ligand activation of ion channels in normal pituitary cells comes from the work of Inenaga & Mason who studied the effect of $\gamma$-aminobutyric acid (GABA) on a $GABA_a$ receptor–$Cl^-$ channel complex in normal bovine lactotrophs (Fig. 5) (Inenaga & Mason, 1987). Likewise, Kehl *et al.* (1987) and Taleb *et al.* (1987) have shown GABA to activate chloride channels in rat pars intermedia cells. GABA has been shown to inhibit PRL release from lactotrophs *in vitro* (Enjalbert *et al.* 1979; Grossman, Delitala, Yeo & Besser, 1981), and its effects on $Cl^-$ channels may be the underlying mechanism. This type of action is typical of neurotransmitter actions on neurones.

There are a number of ways in which releasing factors might act through SMOCs to alter $[Ca^{2+}]_i$. The intracellular messenger inositol trisphosphate appears to function in some cells by stimulating the release of $Ca^{2+}$ into the cell cytoplasm from intracellular stores. Other more indirect effects could be *via* the closing of $K^+$ channels, in turn depolarizing membrane potential, opening of voltage-dependent $Ca^{2+}$ channels or non-specific cation channels and permitting the influx of $Ca^{2+}$ into the cell cytoplasm. This depolarization may be sufficient to open voltage-activated $Ca^{2+}$ channels, but below the threshold for stimulating action potential firing. These factors may also modulate the opening or closing of voltage-independent $Ca^{2+}$ channels in the cell membrane, again leading to the influx of extracellular calcium. Finally, there may exist effects, *via* changes in intracellular messengers, influencing active pumps or passive ion exchange mechanisms in the plasma and intracellular organelle membranes that normally regulate intracellular $Ca^{2+}$ levels.

We thus can reach a tentative conclusion that under some conditions hypophyseal factors may regulate hormone release *via* changes in membrane potential (GABA actions), whereas other factors may influence release in a non-voltage-

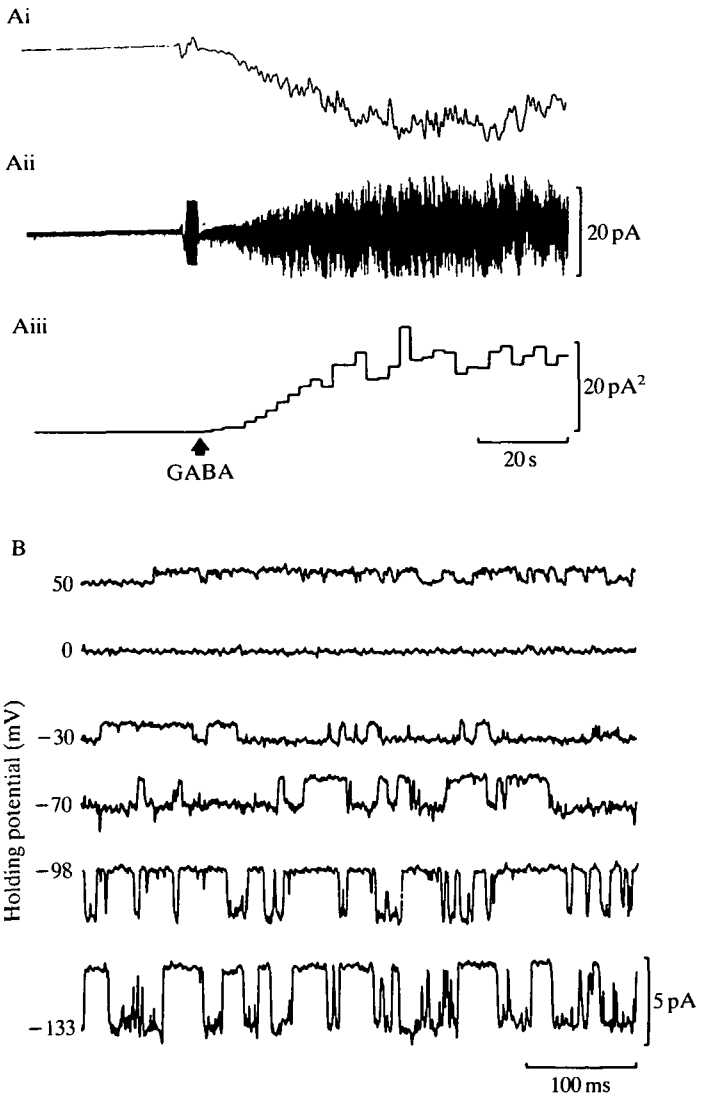



Fig. 5. γ-Aminobutyric acid (GABA)-activated noise in a cultured normal bovine lactotroph, recorded under whole-cell, voltage-clamp in extracellular choline chloride and intracellular CsCl. Applied GABA concentration was 10 $\mu$mol $l^{-1}$, and holding potential was −50 mV. (A) Changes in steady inward current caused by GABA (i), a.c. current filtered at a.c. −800 Hz (ii), and variance of GABA noise, yielding an estimated single-channel current of 0·88 pA under these conditions of symmetrical chloride concentration (iii). (B) GABA-induced single-channel currents in the presence of 100 $\mu$mol $l^{-1}$ GABA recorded in an outside patch of membrane. The conductance of the GABA-activated channel was 23 pS.

dependent manner (e.g. GRF action on GH release, GnRH action on LH release). In the former case these cells behave in a manner normally associated with neurones, whereas the non-voltage-dependent regulation of release is more akin to that in other cells within the periphery. Endocrine cells seem then to possess properties of both these cell types.

### Intracellular modulators – evidence for second-messenger systems

Much evidence with clonal and normal pituitary cells suggests that when cell membrane receptors are stimulated by hypothalamic peptides, changes in resting levels of a variety of soluble cytoplasmic molecules are observed. The formation or release of these molecules occurs through the regulation of membrane-bound enzymes such as adenylate cyclase, guanylate cyclase and phospholipase C. The molecules formed can be grouped under the heading of intracellular messengers, and include the cyclic nucleotides cyclic AMP and cyclic GMP, diacylglycerol, inositol trisphosphate, and $Ca^{2+}$.

One mechanism for intracellular regulation of intracellular events is *via* the phosphorylation of proteins. Many important regulatory molecules such as ion channels and enzymes are proteins, and their activation or inactivation by phosphorylating processes plays a critical role in the control of cell activity. Many of these 'intracellular messengers' appear to activate specific enzymes (such as protein kinases and calcium-binding proteins), which in turn phosphorylate other proteins. An example of the consequences of phosphorylation is that when voltage-activated $Ca^{2+}$ currents are recorded, the currents decrease in amplitude very rapidly as the cytoplasmic contents are dialysed by the pipette solution. However, this $Ca^{2+}$ current rundown is to a large extent prevented if millimolar concentrations of MgATP are included in the pipette (DeRiemer & Sakmann, 1986; Cobbett *et al.* 1987*b*), or if other high-energy-phosphate-generating enzyme systems are employed.

However, the first electrophysiological evidence that intracellular messenger substances are involved in modulation of anterior pituitary ion channels came from work on gonadotrophin-secreting cells (Mason & Waring, 1985). First, when gonadotrophs were stimulated to release LH or FSH by the hypothalamic peptide GnRH, intracellular recordings showed that action potentials were not generated, the membrane was only slightly depolarized (by 2–5 mV) and, significantly, that membrane voltage 'noise' increased markedly – implying the activation of underlying ion channels in the absence of voltage changes. Second, when single $Ca^{2+}$ channels from an isolated membrane patch attached to the gonadotroph were recorded, GnRH applied to the membrane *outside* the patch caused activation of $Ca^{2+}$ channels *inside* the patch (Fig. 6A). GnRH applied directly to the extracellular face of the membrane patch inside the electrode did not activate channels. Third, if the patch was excised from the cell after GnRH had been observed to activate $Ca^{2+}$ channels, the channel activity rapidly disappeared (Fig. 6B), even if $Ca^{2+}$ concentration on the cytoplasmic face was elevated to very

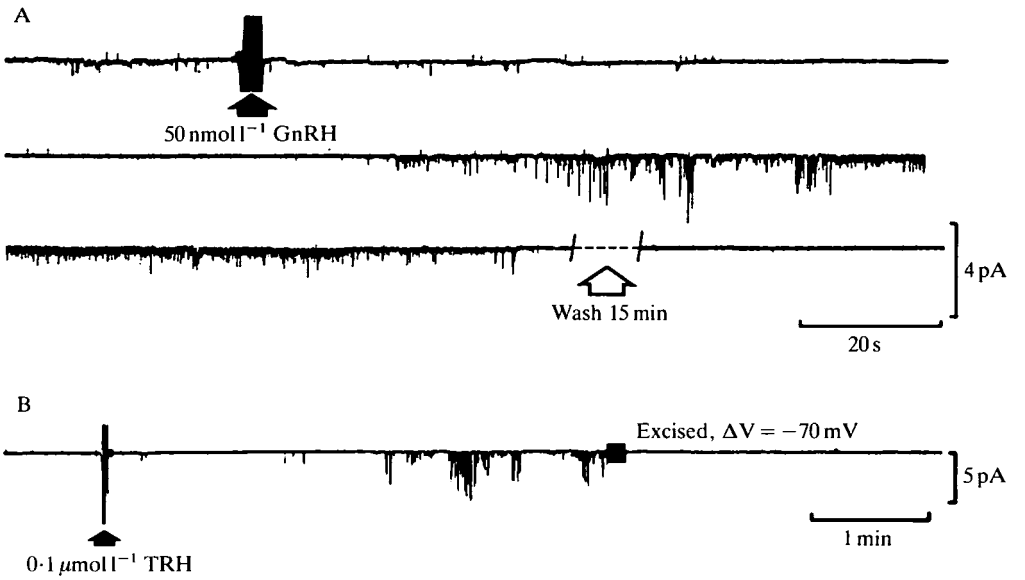



Fig. 6. Single-channel membrane current recordings from cell-attached recordings from a gonadotroph (A) and a lactotroph (B) during stimulation with gonadotrophin-releasing hormone (GnRH) and thyrotrophin-releasing hormone (TRH), respectively. The cells were maintained in a normal physiological medium and the recording pipette contained $BaCl_2$. Note that channel openings occur following application of the releasing hormone but that the action is mediated by an intracellular mechanism since the releasing hormone is not in contact with the recorded patch. The effect of releasing hormone may be reversed by removing it from the external medium by washing or by excision of the patch from the cell so that the internal surface of the membrane is no longer in contact with the cytoplasm. (Reprinted from Mason *et al.* 1986, with permission.)

high levels. This evidence taken together suggested that GnRH probably caused the formation of intracellular messenger substance(s) which acted on the cytoplasmic membrane face to open calcium-selective channels, and that the messenger substance in gonadotrophs was something other than $Ca^{2+}$.

Other more recent work using whole-cell recording to dialyse single pituitary cells has shown that the hypothalamic peptides GnRH, GRF and TRH will not, in general, activate their target cell if the cell interior is perfused, and this has been taken to indicate that soluble messengers are removed from the intracellular milieu during this process. Some good evidence for this has been the recent work of Dufy, Jaken & Barker (1987) who showed that dialysed GH3 cells would not respond to TRH unless the pipette contents contained both protein kinase C (PKC) and 100 nmol $l^{-1}$ $Ca^{2+}$ (but not if $Ca^{2+}$ was buffered to 10 nmol $l^{-1}$), suggesting a possible synergy of action in supporting action potential firing (Fig. 7).

How then do these intracellular messengers regulate ion channels in anterior pituitary cells, and what is their role in secretion?

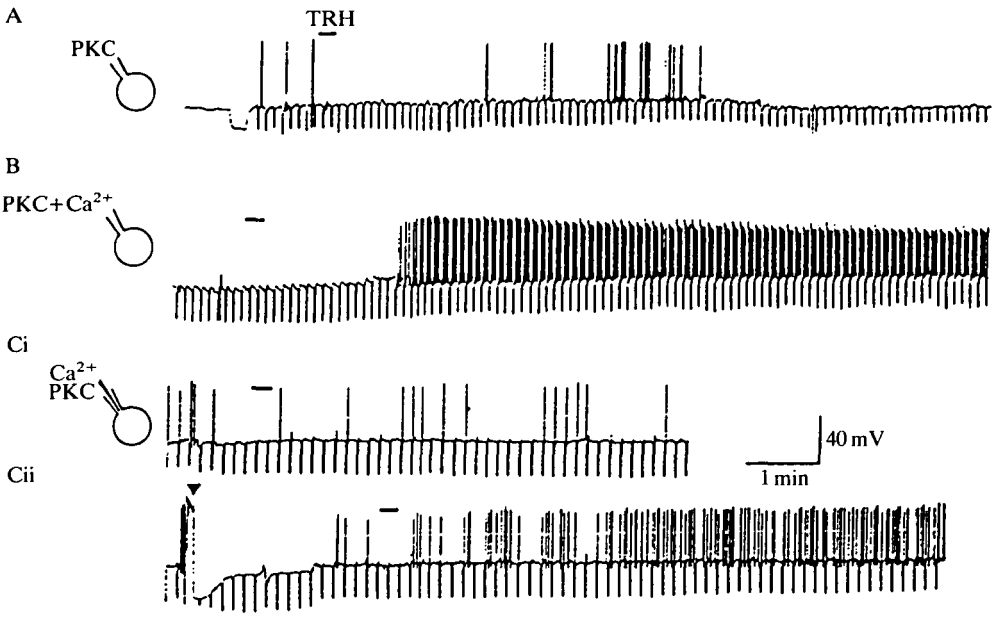



Fig. 7. Thyrotrophin-releasing hormone (TRH)-induced action potential activity in protein kinase C (PKC)-dialysed cells is $Ca^{2+}$-dependent. Whole-cell recordings of three GH3/B6 cells were carried out with 1 ng $ml^{-1}$ PKC included in the patch pipette. (A) At about 10 nmol $l^{-1}$ $[Ca^{2+}]_i$, 50 nmol $l^{-1}$ TRH triggers a barely detectable depolarization and several bursts of action potentials. (B) With $[Ca^{2+}]_i$ buffered to about 50–100 nmol $l^{-1}$, TRH induces a gradual depolarization of about 10 mV and irreversible high-frequency action potential activity. (C) The recording has been made with a $Ca^{2+}$-containing cannula inserted far down in the patch pipette, which includes 0·5 ng $ml^{-1}$ PKC in the potassium gluconate electrolyte (resting potential, −42 mV). (Ci) As in A, TRH has little, if any, effect on membrane properties when the cell is dialysed with PKC at an estimated $[Ca^{2+}]_i$ of 10 nmol $l^{-1}$. PKC/electrolyte buffered at 100 nmol $l^{-1}$ $[Ca^{2+}]_i$ has been expressed by pressure from the tip of the cannula in the patch pipette. Following the artefact there is a 20-mV hyperpolarization associated with a marked increase in conductance that lasts about 30 s, after which the cell repolarizes to −47 mV. Injection of depolarizing current, bringing the cell to −42 mV, reveals the presence of more low-amplitude voltage fluctuations than previously observed. TRH now induces action potential activity. (From Dufy, Jaken & Barker, 1987.)

### Are intracellular $Ca^{2+}$ changes dependent on membrane voltage changes?

Work on gonadotrophs suggested (Mason & Waring, 1985, 1986) that $Ca^{2+}$ channels in the plasma membrane activated by GnRH were not dependent on voltage, but rather it was inferred that channel activation occurred, at least in part, because of modulation by some soluble intracellular messenger. A more direct test of this hypothesis is now possible, using patch-clamp methodology to both clamp the membrane voltage and dialyse the cell interior with a $Ca^{2+}$-sensitive dye (Indo-1) allowing intracellular $Ca^{2+}$ concentration to be monitored by microspectro-

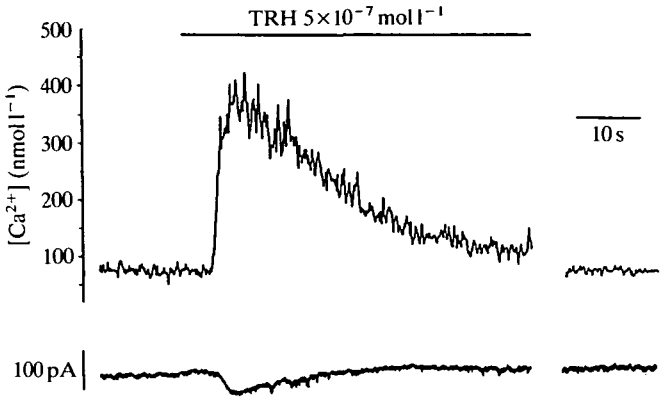



Fig. 8. Calcium transient in response to thyrotrophin-releasing hormone (TRH) in a voltage-clamped GH3 cells. Upper trace shows ratio of indo-1 fluorescence displayed to give a linear $Ca^{2+}$ scale. Lower trace shows membrane current; holding potential was −70 mV. Cell input resistance was 0·7 GΩ. Resting $Ca^{2+}$ level was close to 100 nmol l$^{-1}$ in this cell. On TRH application $[Ca^{2+}]$ rose after a delay (difficult to quantify as both applied) to about 350 nmol l$^{-1}$ and then declined in the continued presence of the agonist to a level above basal. TRH was washed off during the gap in the traces (150 s). $[Ca^{2+}]$ returned to basal level during this time. The rise in $[Ca^{2+}]$ was associated with an inward current at −70 mV which is probably a $Ca^{2+}$-activated chloride current. With a physiological chloride reversal potential more negative than the 0 mV in this experiment one might expect an outward $K^+$ current to predominate, resulting in the initial hyperpolarization in response to TRH seen with microelectrode recordings in these cells. Bathing solution was 1 mmol l$^{-1}$ $Ca^{2+}$-containing saline solution. Pipette solution was (in mmol l$^{-1}$): KCl, 140; Hepes, 10; $MgCl_2$, 4; NaATP, 2; NaGTP, 0·2; indo-1 potassium salt, 0·05; pH 7·2 with KOH. Temperature 24 °C. Photon counts were sampled every 50 ms simultaneously at 480 and 405 nm and used to calculate a ratio after background subtraction. Ratio values were converted to $[Ca^{2+}]$ values using the method of Grynkiewicsz, Poenie & Tsien (1985).

fluorimetry. This combined technique has been used to study the action of TRH in single prolactin-secreting GH3 cells held under voltage-clamp.

Under these conditions, a depolarizing voltage step pulse clearly activates a rise in intracellular $Ca^{2+}$ concentration (Benham, 1988). For instance, in the presence of only 1 mmol l$^{-1}$ extracellular $Ca^{2+}$, resting intracellular $Ca^{2+}$ level rises from about 100 to about 400 nmol l$^{-1}$ during a 0·5-s voltage pulse which evokes an inward $Ca^{2+}$ current and decays over about 3–6 s. This demonstrates the presence of voltage-activated $Ca^{2+}$ channels in the plasma membrane.

Fig. 8 shows the results of TRH application when membrane voltage is maintained close to resting potential, at −70 mV, with 1 mmol l$^{-1}$ extracellular calcium. In this experiment, a large transient rise in intracellular $Ca^{2+}$ from about 80 to 400 nmol l$^{-1}$ followed by a sustained plateau level slightly above the basal value was observed, despite the fact that membrane potential did not change. A small transient inward current was also noted, which may have been a $Ca^{2+}$-activated chloride current. In another experiment, smaller $Ca^{2+}$ transients also

occurred when all extracellular $Ca^{2+}$ had been removed, suggesting the source of the rise in intracellular $Ca^{2+}$ concentration to be partly intracellular, but also with a significant contribution from plasma membranes which are probably activated close to membrane resting potential.

## Cyclic AMP

Accumulation of intracellular cyclic AMP following adenylate cyclase activation in pituitary cell cultures has been observed following activation by such factors as GnRH, GRF and vasoactive intestinal peptide (VIP) (Borgeat *et al.* 1972; Kaneko *et al.* 1973; Bilezikjian & Vale, 1983; Naor, Snyder, Fawcett & McCann, 1980), and is associated with gonadotrophin and growth hormone release. How then is the rise in cyclic AMP level coupled to hormone release in these cells?

Recent studies in our laboratory on ovine gonadotrophs indicate that cyclic AMP could influence the release process by changing cellular excitability through alteration of ionic channel gating. In particular, we have focused on $Ca^{2+}$-dependent $K^+$ channels of about 100 pS conductance, which probably play a major role in control of membrane potential during rises in intracellular $Ca^{2+}$ concentration (Fig. 9A). Activation of the adenylate cyclase enzyme by forskolin applied extracellularly causes closure of $Ca^{2+}$-dependent $K^+$ channels, shown using cell-attached, patch-clamp recordings. Elevation of intracellular cyclic AMP concentration by dibutyryl cyclic AMP (a membrane-permeable analogue of cyclic AMP) produces similar results (Fig. 9B). The fact that this is a direct action of cyclic AMP on the intracellular face of the membrane was confirmed by application of cyclic AMP to the cytoplasmic face with recordings from inside-out patches.

We have additionally explored the biochemical basis for this inhibition and found it to occur as a result of a two-stage process. First, the cyclic AMP binds to a cyclic AMP-dependent protein kinase (cAMPPK) present in the membrane. This kinase has two components, a regulatory subunit which is bound by cyclic AMP and causes its detachment from the second, catalytic, subunit. The latter phosphorylates the $Ca^{2+}$-dependent $K^+$-channel protein and thus closes it. Direct application of the catalytic subunit of the cyclic AMP-dependent protein kinase (CS-cAMPPK) to inside-out patches decreased channel activity in a manner similar to that of cyclic AMP, and this can be reversed by the protein kinase inhibitor applied on the cytosolic face (Fig. 10B) following closure of the channel by $10\,\mu mol\,l^{-1}$ cyclic AMP (Fig. 10A). As for hormone secretion, cyclic AMP potentiates the release of LH in response to GnRH, probably by causing a shift in the membrane potential to a more depolarized state, since $K^+$ channels normally participate in maintaining the cell in a hyperpolarized state. This would ensure further $Ca^{2+}$ influx through voltage-activated $Ca^{2+}$ channels leading to more hormone release (Sikdar *et al.* 1987).

## Cyclic GMP

The second-messenger role of cyclic GMP in the secretory phenomenon is less

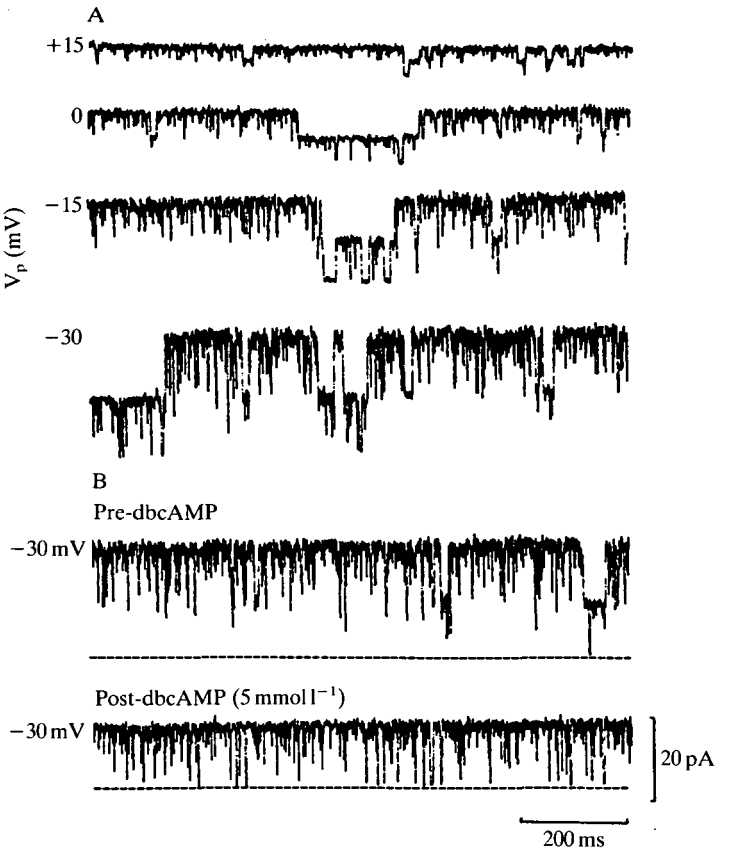



Fig. 9. Effect of dibutyryl cyclic AMP (dbcAMP) in a cell-attached, patch-clamp recording of two channels in the membrane of an ovine pituitary gonadotroph. The patch pipette was filled with (in mmol $l^{-1}$) KCl, 130; Hepes, 5; glucose, 10; and the bath contained NaCl, 130; KCl, 5; Hepes, 5; $CaCl_2$, 5; glucose, 10; pH 7·4 with Trizma base. (A) Dependence of channel current amplitude on pipette potential, $V_p$. (B) Closure of one channel in the patch by 5 mmol $l^{-1}$ dibutyryl cyclic AMP, indicating a general decrease in the probability of channel opening catalysed by cyclic AMP.

than clear. For instance, although GnRH stimulates the formation of cyclic GMP in cultured pituitary cells, increased cyclic GMP production provoked by sodium nitroprusside does not lead to increased LH release (Naor & Catt, 1980), suggesting that cyclic GMP production is not an obligatory step in LH release. In rat somatotrophs, however, cyclic GMP stimulates GH secretion (Peake, Steiner & Daughaday, 1972). We have recently found in cell-attached, patch-clamp recordings that cyclic GMP, presumably *via* a protein kinase molecule, opens a $Ca^{2+}$-permeable channel in ovine gonadotrophs, similar to the action of GnRH. Cyclic GMP clearly acted on these channels at the cytoplasmic face in inside-out patches, but its overall role in modulation of hormone release remains obscure at present.

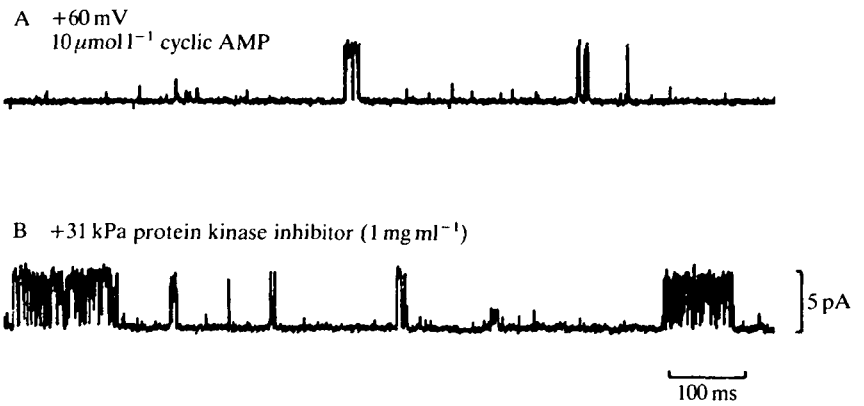



Fig. 10. Increase in activity of a $Ca^{2+}$-activated $K^+$ channel following pressure ejection of protein kinase inhibitor onto the intracellular face of the patch membrane in an inside-out patch. The patch pipette was filled with (in mmol $l^{-1}$) KCl, 130; Hepes, 5; glucose, 10; and the bath contained KCl, 130; Hepes, 5; glucose, 5; $CaCl_2$, 1; EGTA, 10; MgATP, 1; and cyclic AMP, 0.01. The enzyme obtained from Sigma had an activity consistent with 1 μg of protein inhibiting 0·02 phosphorylating units of cyclic-AMP-dependent protein kinase. In A the large channel has been inhibited by the addition of cytosolic cyclic AMP, and in B pressure application of the protein kinase inhibitor has caused the channel to open with increased probability.

### The phosphatidylinositol system

The key enzyme activating the phosphatidylinositol system is phosphoinositidase C (PIC), which causes the formation of phosphoinositides (such as Ins$P_3$ and Ins$P_4$) as well as diacylglycerol (DAG). In anterior pituitary cells the factors known to stimulate this system include acetylcholine (ACh) action on muscarinic receptors, bombesin and, as described below, TRH.

TRH causes hydrolysis of a phosphoinositide precursor, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$] into cytoplasmic (Ins$P_3$) and membrane-bound (DAG) components. The concentration of Ins$P_3$ reaches a maximum in 15 s (Rebecchi & Gershengorn, 1983) and possibly contributes to an initial rise of intracellular $Ca^{2+}$ level following TRH activation. The membrane-bound component, DAG, activates protein kinase C (PKC) which is thought to cause a second phasic rise in intracellular $Ca^{2+}$ level based on findings in the GH cell. These events may play a role in transduction of the TRH signal into a secretory response.

Electrophysiological recordings of bovine lactotrophs and GH3/B6 cells using conventional intracellular recording techniques, reveal that TRH induces an increase in $Ca^{2+}$-dependent $K^+$ conductance leading to cell hyperpolarization, followed by a period of sustained spiking activity but with only very slight depolarization (Ingram, Bicknell & Mason, 1986; Ozawa & Kimura, 1979). Phorbol myristyl acetate (PMA) mimics the TRH response (Dufy *et al.* 1987) presumably by activation of PKC. However, the electrophysiological responses to

both TRH and PMA were lost when the whole-cell recording configuration of the patch-clamp technique was employed – presumably because of the dialysis of important compounds from the cell cytoplasm. The responses were restored, however, when products of cell lysates were included in the patch electrode. The active cellular components needed to produce the response were identified as protein kinase C and Ins$P_3$. The phosphoinositide Ins$P_3$, or calcium, was necessary to allow the TRH-induced hyperpolarization, and PKC, in combination with calcium, allowed the TRH stimulation of the slight depolarization and increase in spiking rate. PKC alone in the pipette failed to elicit either the TRH or the PMA electrical response, implying an essential role of $Ca^{2+}$ in the TRH-stimulated electrophysiological response. In fact, protein kinase C and $Ca^{2+}$ synergistically increase GH, LH and TSH secretion from anterior pituitary cells (Judd, Koike, Yasumoto & MacLeod, 1986).

## Calcium

$Ca^{2+}$ entering the cell cytoplasm can activate a number of $Ca^{2+}$-binding proteins such as calmodulin (CaM), which is associated with a number of intracellular events provoked by a rise in intracellular $Ca^{2+}$ concentration (Cheung, 1980). The $Ca^{2+}$–CaM complex can then act directly on other proteins, e.g. the ATPase pump found in many cell membranes, that pumps $Ca^{2+}$ out of the cell, or indirectly *via* phosphorylation of other enzymes such as the protein kinases.

In gonadotrophs, the physiological releasing factor GnRH induces redistribution of calmodulin from the cytosol to the plasma membrane (Conn, Chafouleas, Rogers & Means, 1981), and there is evidence for an association between calmodulin and membrane patch areas containing receptors for GnRH (Jennes, Bronson, Stumpf & Conn, 1985). However, the cellular protein kinases, which bridge the events between calmodulin and cytoskeletal activation and translate these to granule movement, extrusion and exocytosis, still remain to be characterized. Hormone release from the pituitary seems to be dependent on calmodulin since it can be attenuated by antagonists of calmodulin (Conn, Rogers & Sheffield, 1981).

$Ca^{2+}$ also has other actions, independent of calmodulin, on other $Ca^{2+}$-binding proteins. For example, it can act directly upon the $Ca^{2+}$-dependent $K^+$ channel to cause its opening and thus influence cell excitability as described above.

There exist many interrelationships between these intracellular messengers in the cell. For example, cyclic AMP can act to influence the influx or extrusion of $Ca^{2+}$ from the cytoplasm. $Ca^{2+}$ may act, *via* calmodulin, to activate or inactivate adenylate cyclase (which produces cyclic AMP) or phosphodiesterase (which breaks down cyclic AMP). In smooth muscle cells the ability of the $Ca^{2+}$–CaM complex to phosphorylate (and activate) myosin light chain kinase (MLCK) is inhibited by prephosphorylation of another site on MLCK by cyclic AMP-dependent protein kinase.

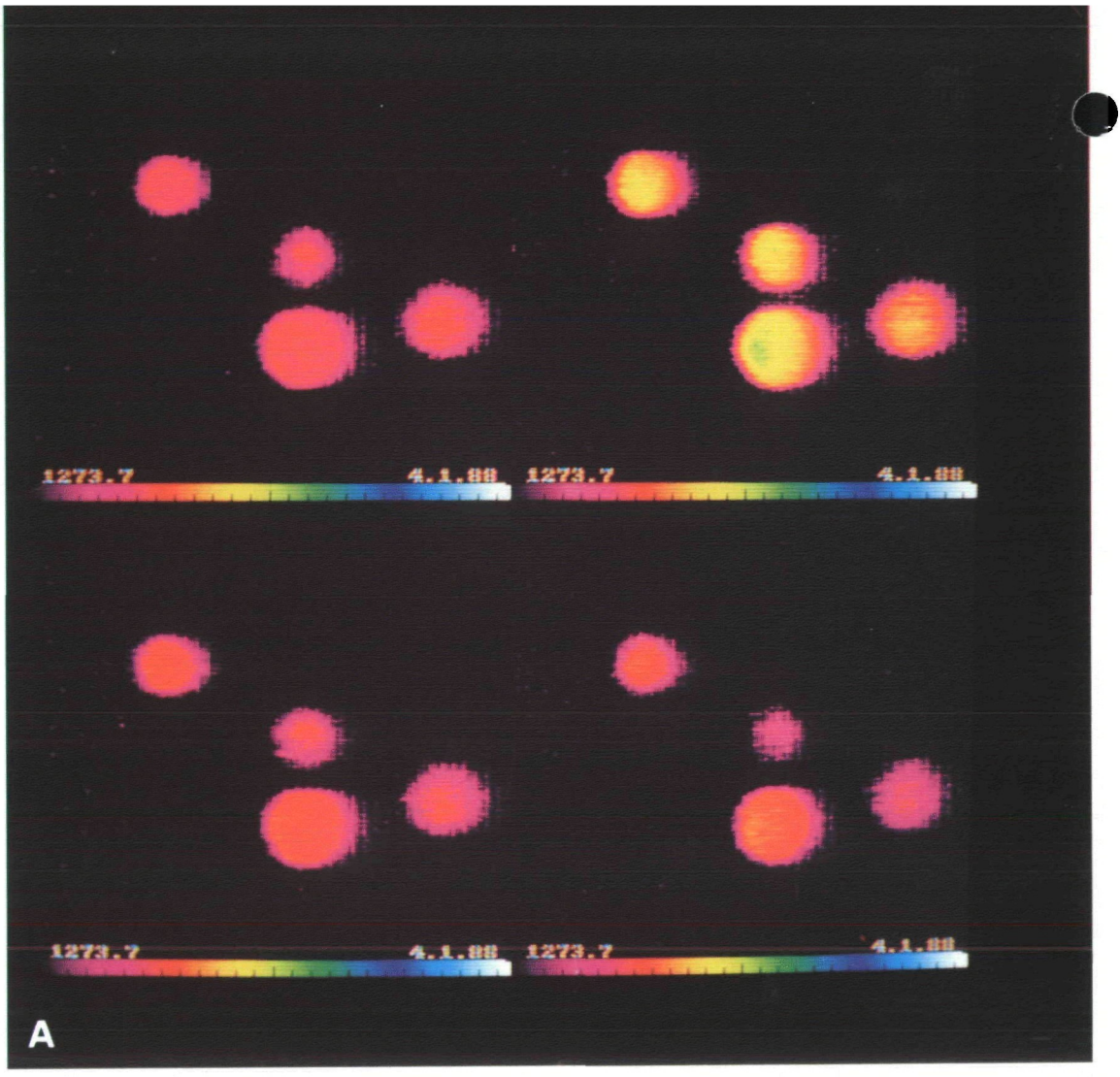
In anterior pituitary cells an example of the interrelationship between cyclic

AMP and the phosphatidylinositol system has been suggested to occur in somatotrophs (Ingram & Bicknell, 1986). The GRF receptor is known to be coupled to adenylate cyclase and stimulation of the receptor with GRF leads to a rise in intracellular cyclic AMP concentration. TRH, however, acts *via* the stimulation of PIC. Application of GRF and TRH together in these cells will release very significantly more GH than the sum of the individual releasing factors alone, implying that activation of more than one intracellular pathway may promote, in this case, a synergistic response. Another interrelationship of intracellular messenger action in anterior pituitary cells is seen with the $Ca^{2+}$-dependent $K^+$ channel, which is activated by $Ca^{2+}$ but closed by cyclic AMP.

How do these anterior pituitary cells differ from one another? It seems likely that release-modifying factors act on different cells in a similar manner, e.g. the TRH stimulation of lactotrophs and thyrotrophs is *via* rises in $InsP_3$ and DAG in both cell types. What may be different, however, is the fact that each cell type within the pituitary has its own receptors for specific peptides and non-peptides, and the interrelationship between these extracellular and intracellular stimuli will be unique for a particular cell. However, it seems likely that a common action is to change the concentration of $Ca^{2+}$ inside the cell, enabling the role of $Ca^{2+}$ as a second messenger and allowing it to act in synergy with PKC and other systems.

## Calcium and hormone secretion

Anterior pituitary hormone secretion is a $Ca^{2+}$-dependent process. Depriving cells of extracellular $Ca^{2+}$ by incubation in media containing $Ca^{2+}$ buffers such as EGTA inhibits GnRH-induced LH secretion (Bates & Conn, 1984), GRF-induced GH secretion (Sheppard, Kraicer & Milligan, 1980) and TRH-induced PRL secretion (Thorner, Hackett, Murad & MacLeod, 1980). Furthermore, this inhibition can be reversed by elevation of extracellular calcium. Agonist-induced hormone secretion can be inhibited by various $Ca^{2+}$ channel blockers such as cobalt and verapamil (Conn, McArdle, Andrews & Huckle, 1987; Merritt & Brown, 1984; Ingram *et al.* 1986). Secretion of LH, GH and PRL can be enhanced in several ways, such as by the $Ca^{2+}$ ionophore A23187 (Sheppard *et al.* 1980; Conn, Rogers & Sandhu, 1979; MacLeod, Schettini & Canonico, 1984), by raising the extracellular potassium concentration to depolarize the cell membrane and promote influx through voltage-dependent $Ca^{2+}$ channels (Sheppard *et al.* 1980; Hopkins & Walker, 1978; Nakano, Fawcett & McCann, 1976) or by maitotoxin, a $Ca^{2+}$ channel activator (Schettini *et al.* 1984; Rasmussen & Barret, 1984). In normal bovine lactotrophs, the $Ca^{2+}$ channel blockers ($Co^{2+}$, $Mn^{2+}$) decrease the basal and TRH-stimulated secretion (Cobbett *et al.* 1987*b*). This and other evidence strongly suggests that alterations of intracellular ionized $Ca^{2+}$ concentration may also play a major role in anterior pituitary cell stimulus–secretion coupling. Further, other experiments also suggest that intracellular $Ca^{2+}$ may serve to couple the different signal transduction pathways present in these cells.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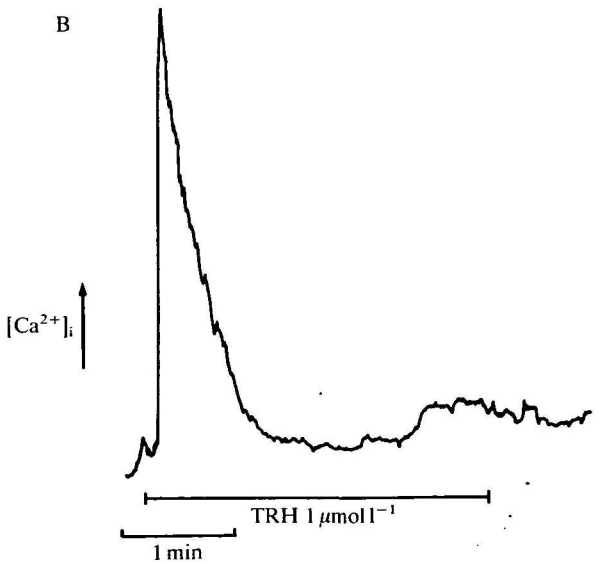
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Fig. 11

**Intracellular free $Ca^{2+}$ changes in anterior pituitary cells**

Using the photoproteins aequorin and quin2 (Tsien *et al.* 1982; Grynkiewicz *et al.* 1985) it has been shown that TRH stimulates a rapid elevation of intracellular $Ca^{2+}$ concentration in suspensions of GH3 cells (Gershengorn, 1985) and bovine pituitary cells (Schofield, 1983). Several studies have shown that the increase is biphasic. The first phase consists of a rapid several-fold transient increase which peaks within 6–8 s and returns rapidly towards prestimulation levels. The increase is unaffected by prior removal of extracellular $Ca^{2+}$ or the $Ca^{2+}$ channel blockers nifedipine and verapamil, and is therefore believed to be the result of release from intracellular stores. This is followed by a prolonged phase dependent on the influx of external $Ca^{2+}$ since it is abolished by the removal of extracellular $Ca^{2+}$ and is markedly reduced by nifedipine and verapamil (Albert & Tashjian, 1984; Gershengorn & Thaw, 1985). Similar changes in gonadotrophs are stimulated by GnRH (Limor *et al.* 1987).

A similar biphasic pattern of PRL and GH secretion in response to TRH has been observed. The transient phase involves the release of stored hormone and appears to depend on $Ca^{2+}$ mobilization from intracellular stores, whereas the second phase, involving the release of newly synthesized hormone, is dependent on $Ca^{2+}$ influx from outside the cell (Aizawa & Hinkle, 1985). It is believed that TRH binding to its receptor stimulates PtdIns(4,5)$P_2$ hydrolysis to Ins(1,4,5)$P_3$ and DAG. The former then diffuses to a non-mitochondrial intracellular $Ca^{2+}$ store and causes the first-phase transient rise of intracellular $Ca^{2+}$ concentration which in turn leads to the release of stored hormone. It is also suggested that TRH binding initiates an inhibitory process involving PKC that rapidly reverses the rise in intracellular ionized $Ca^{2+}$ level. The second phase of extracellular $Ca^{2+}$ influx is believed to be responsible for the prolonged phase of GH and PRL secretion, although the mechanisms and pathways involved remain largely unresolved.

We have recently begun to monitor intracellular free $Ca^{2+}$ concentration at the single-cell level in enriched cultures of normal bovine lactotrophs using the technique of fura2 ionized $Ca^{2+}$ imaging (Fig. 11A), which provides a pixel-by-pixel dynamic image of intracellular ionized $Ca^{2+}$ concentration. Challenging cells with 10–100 nmol $l^{-1}$ TRH, maximal for PRL secretion, induces a rapid transient

Fig. 11. Thyrotrophin-releasing hormone (TRH) causes a transient increase of intracellular free calcium in bovine lactotrophs. (A) Fluorescence ratio images of TRH-induced changes of intracellular calcium level. The sequence shows cells prior to stimulation (top left), at 20, 30 and 120 s after application of TRH (1 $\mu$mol $l^{-1}$). Cells were loaded with fura-2/AM for 30 min. The medium used was the same as the bathing medium used for capacitance measurements (see Fig. 12). The cells were alternately excited with 340 and 380 nm light every 40 ms by means of a rotating mirror. Emission at 500 nm was detected by an intensified CCD camera and the ratio of emissions at the two excitation wavelengths, 340/380, calculated on a pixel-per-pixel basis (512×512), stored on video tape and subsequently displayed with a pseudocolour logarithmic scale. (B) A plot of total intracellular free calcium concentration against time in one of the above cells, recorded as the change of light intensity of the black and white ratio image by means of a photodiode to which the whole cell image was projected by a lens.

increase of intracellular $Ca^{2+}$ concentration in most cells within seconds of TRH application (Fig. 11A,B). This consists of a several-fold increase with a rise time of the order of 1 to several seconds followed by an exponential decay which lasts between 8 and 25 s. Similar transient responses have also been observed in the presence of extracellular cadmium and cobalt or when extracellular $Ca^{2+}$ was substantially reduced just prior to stimulation. This is strong evidence that the transient response is the result of $Ca^{2+}$ release from intracellular stores. These results are in general agreement with the first-phase $Ca^{2+}$ response reported in GH3 cells.

In summary, factors acting to cause hormone release from anterior pituitary cells also cause increases in free cytoplasmic $Ca^{2+}$ concentrations. Is this $Ca^{2+}$ rise linked directly to secretion? To begin to answer these questions we have employed new techniques to record the secretory event 'exocytosis', and the preliminary results we, and others, have obtained are described in the following section.

**Exocytosis, capacitance measurements and stimulus–secretion coupling**

Exocytotic release is a fundamental process in the function of neurones and other secretory cell types. Secretory products (neurotransmitters, hormones, various peptides, proteins, purinergic substances, etc.) are packed in vesicles. A number of these vesicles fuse with the surface membrane, during both non-activated and activated phases, to release secretory product into the extracellular space. This process is called exocytosis (see review by Knight & Baker, 1987). In addition to regulated exocytosis that is triggered by a stimulus, continuous fusion of vesicles with the plasma membrane probably occurs. This constitutive process is thought to be part of the continuous cycling of cell membranes.

Stimulated secretion from nerve terminals as well as many other cells requires extracellular $Ca^{2+}$ (Douglas, 1968). Katz & Miledi (1967) first suggested that presynaptic action potentials open $Ca^{2+}$ channels in the presynaptic terminal, causing an influx of $Ca^{2+}$, which triggers the secretory machinery. This proposal has been extended from neural tissue to other secretory cells, where extracellular $Ca^{2+}$ is essential for long-term release of hormone.

A major obstacle to elucidating the mechanism of the stimulus–secretion pathway is that the events controlling exocytosis are occurring at the inner face of the plasma membrane, which is relatively inaccessible to experimental manipulation. However, the technique of measuring cell membrane capacitance developed by Neher & Marty (1982) enables measurements on a single cell during exocytosis. Such recording is usually made in a whole-cell, patch-clamp recording configuration, in which the intracellular space is dialysed with the solution used to fill the patch pipette. Membrane potential is 'clamped' to a desired level to which an additional sinusoidal voltage is added. Analysis of the resulting sinusoidal current, that flows through the membrane, can reveal conductive and capacitive properties of the cell membrane using a phase-sensitive detector.

**Messenger systems that trigger exocytosis**

Is elevated $[Ca^{2+}]_i$ necessary to trigger exocytosis? Although calcium ions play a pivotal second messenger role in many secretory cell types (Knight & Baker, 1987), there is growing evidence for a number of cells that elevated $[Ca^{2+}]_i$ alone cannot fully explain the observed secretory response. For example, in the platelet, secretion is only triggered by doses of the $Ca^{2+}$ ionophore ionomycin which raise $[Ca^{2+}]_i$ to micromolar levels. Sub-micromolar levels of $Ca^{2+}$ do not trigger secretion (Rink & Hallam, 1984). The natural agent thrombin, however, only raises $[Ca^{2+}]_i$ to sub-micromolar levels ($0{\cdot}1\,\mu mol\,l^{-1}$) but does not trigger secretion. Furthermore, collagen-triggered secretion occurs with no detectable change in $[Ca^{2+}]_i$ from quin2-loaded platelets (Rink, Sanchez & Hallam, 1983). The fact that secretory exocytosis need not be regulated by a rise in $[Ca^{2+}]_i$ is demonstrated in tissues such as juxtaglomerular apparatus (Keeton & Campbell, 1981) and parathyroid gland (Nemeth & Scarpa, 1987). In these cells a rise in $[Ca^{2+}]_i$ is inhibitory to secretion. In addition, protein kinase C will activate physiological processes related to secretion at very low levels of cytosolic $Ca^{2+}$ (DiVirgilio, Lew & Pozzan, 1984).

In the case of bovine lactotrophs our preliminary experiments have shown that it is possible to induce cell degranulation (measured as an increase in capacitance) by dialysing the intracellular space with solutions containing raised $[Ca^{2+}]_i$, (Fig. 12A), and to observe small step-wise fluctuations in membrane capacitance consistent with both fusion and retrieval of single vesicles (see Fig. 12B). Assuming the specific membrane capacitance of the lactotroph cell membrane to be about $1\,\mu F\,cm^{-2}$, these step changes of about 5 fF reflect fusion (or retrieval) of granules with diameters of $0{\cdot}5\,\mu m$, similar to the diameters of granules observed in these cells with electron microscopy. Using pipettes filled with a solution known to support exocytosis in chromaffin cells (Neher & Marty, 1982) and elevating $[Ca^{2+}]_i$ by activating voltage-dependent $Ca^{2+}$ currents is not sufficient to evoke time-locked capacitance changes (a measure of exocytosis), which seems to suggest that other second-messenger systems in addition to $Ca^{2+}$ are probably involved in controlling secretion in these cells. There is other evidence which supports this tentative speculation. In GH3 cells it was observed that the sustained phase of secretion is $Ca^{2+}$-independent (Gershengorn, 1986); in rabbit neutrophils secretion can be stimulated at extremely low levels of $[Ca^{2+}]_i$ ($<10^{-10}\,mol\,l^{-1}$) (Barrowman, Cockcroft & Gomperts, 1986). Furthermore, using the whole-cell, patch-clamp technique, it was found that mast cells would not generally degranulate even if $[Ca^{2+}]_i$ was maintained in the range $0{\cdot}2$–$10\,\mu mol\,l^{-1}$ (Fernandez, Neher & Gomperts, 1984).

The unifying factor linking all of these examples is that second messengers other than $Ca^{2+}$ could play an equally important role in stimulus–secretion coupling. Possible candidates could include products of phosphoinositide metabolism, cyclic nucleotides, guanine-nucleotide-binding proteins (G-proteins), and other molecules as yet undefined. In the example of the platelet the available evidence suggests that it is a product of phosphoinositide breakdown, diacylglycerol, which

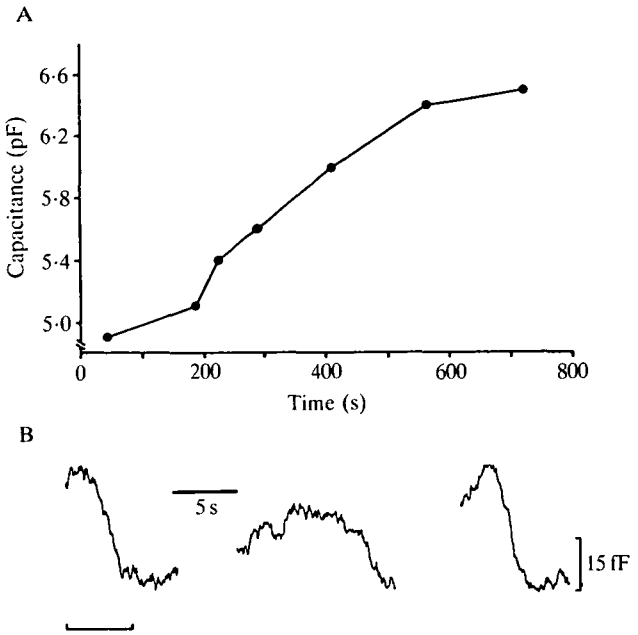


Fig. 12. Capacitance measurements in a single bovine lactotroph grown in cell culture. (A) Capacitance ($C_m$) increase in a single lactotroph recorded with the tight-seal, whole-cell recording technique. At time zero, the membrane patch beneath the pipette tip was ruptured by a brief pulse of suction. This established both diffusional and electrical contact between the pipette and cell interior. The cell was voltage-clamped at −66 mV. Points represent $C_m$ measurements obtained by nulling out the capacitive current evoked with a sine wave signal (1600 Hz, 12·5 mV; summed to the holding potential) by means of the capacitance cancellation control on the patch-clamp apparatus (designed by F. Henigman). Pipette filling solution was (in mmol $l^{-1}$): potassium gluconate, 140; NaCl, 10; $MgCl_2$, 2; Hepes/Trizma baze (Sigma), 10; $K_2H_2EGTA$, 0·1; $CaH_2EGTA$, 0·5; pH 7·2. The cells were bathed in a medium containing (in mmol $l^{-1}$): NaCl, 127; KCl, 5; $MgCl_2$, 2; $NaH_2PO_4$, 0·5; $NaHCO_3$, 5; $CaCl_2$, 5; Hepes/NaOH, 10; bovine serum albumin, 0·1 %; pH 7·2. Both solutions were iso-osmolar. The slow increase in $C_m$ was associated with an apparent degranulation of the cell, when viewed under the microscope. (B) Sections of the capacitance signal recorded with the use of the lock-in amplifier in a cell after the slow increase of $C_m$ had reached a plateau. Upward deflections denote an increase in capacitance. Note the step changes that are believed to represent insertions and retrieval of single vesicles. Recording configuration is the same as above; this cell was voltage-clamped at −61 mV.

plays a key role in the secretory response, presumably by activating the enzyme PKC (Nishizuka, 1984). In the case of mast cells, degranulation initiated by the addition of GTP analogues (in the presence of ATP) was sufficient to stimulate secretion (Fernandez *et al.* 1984). In these cases it is likely that the main role of the additional second messenger is to modulate the $Ca^{2+}$-sensitivity of the exocytotic machinery since buffering of $[Ca^{2+}]_i$ to very low levels in platelets resulted in the

failure of PKC activators to trigger secretion (Knight, 1986), and a rise in $[Ca^{2+}]_i$ accelerated, but did not initiate, the degranulation of mast cells, judged from the capacitance signal that is complete in about 3–5 min (Neher, 1988).

Interestingly, rabbit neutrophils can be stimulated to secrete by GTP analogues at $<10^{-10}$ mol $l^{-1}$ $Ca^{2+}$ (Barrowman *et al.* 1986). Under these conditions activation of polyphosphoinositide phosphodiesterase (the enzyme responsible for the production of diacylglycerol and inositol trisphosphate which is under the control of the G-protein, $G_p$) does not occur (Cockcroft, 1986). In this case it is unlikely that secretion is truly $Ca^{2+}$-independent. GTP-analogue-induced $Ca^{2+}$-independent secretion has also been reported in rat insulinoma RINm5F cells (Vallor, Biden & Wollheim, 1987) and in bovine adrenal chromaffin cells (Bittner, Holz & Neubig, 1986). There is evidence for the the existence of pertussis-toxin-sensitive G-proteins in anterior pituitary cells (Journot *et al.* 1987), but whether their activation is a necessary prerequisite for secretion from these cells remains to be investigated.

## Conclusions

A variety of new methods for studying the properties of single cells are providing us with new insight into the control of secretion. In the case of anterior pituitary cells which secrete a wide range of physiologically important hormones, the clear challenge is to understand how brain peptides and neurotransmitters regulate electrical activity (i.e. ionic permeability) of both plasma membrane and intracellular membrane compartments, and how these events are coupled to intracellular biochemistry and $Ca^{2+}$ homeostasis and ultimately to exocytosis and hormone release. Already, several unexpected challenges to conventional dogma have come to light.

First, we now know that rises in intracellular $Ca^{2+}$ concentration may be dynamically modulated by both intracellular $Ca^{2+}$ stores and by extracellular sources. The gates, or channels, controlling $Ca^{2+}$ entry into the cytosol are almost certainly regulated in different ways, although some features may be common. We might class one pathway of control as the conventional voltage-regulated pathway, and the second as chemical control, by ligand–channel complexes or by intracellular messengers. Intracellular messengers almost certainly regulate the intracellular compartment, but it appears they may also modulate plasma membrane $Ca^{2+}$ channels in the absence of any large voltage shifts. Both are probably important in optimizing levels of cell $Ca^{2+}$ under different conditions. This information will enable us to focus on key differences between pituitary cells and neurones in respect of $Ca^{2+}$ entry; for example, peptide agonists, unlike certain transmitters, do not alter membrane potential markedly, although they do affect plasma membrane $Ca^{2+}$ permeability.

Second, agonist binding to plasma membrane receptors activates a diversity of intracellular metabolic cascades. We are discovering that a further class of receptors also exists, these being associated with intracellular binding sites and

some intimately coupled to cytosolic receptors linked to plasma membrane ion channels. In this way, both voltage-activated channels and second-messenger-activated channels may be modulated, with consequences for control of secretion.

Third, using techniques for measuring the electrical consequences of exocytosis, we are beginning to establish in pituitary cells, as found for mast cells secreting histamine, that membrane fusion may depend on factors other than intracellular $Ca^{2+}$ concentration and that the fusion event is not an 'all-or-nothing' process. The ability to dialyse the cell cytosol of anterior pituitary cells with candidates for messenger substances is enabling reconstitution studies which should reveal whether calcium ions are really the prime mover in secretion, or merely a modulator acting in synergy with other molecules – cyclic nucleotides, phosphoinositides and so forth. This area of work is likely to provide us with important information not only about the events preceding secretion but also about what follows in membrane recovery – a much slower event whose dependence on $Ca^{2+}$ is only now beginning to be explored.

Finally, it is clear that we are now faced with more questions and challenges about control of the secretory event than may have been obvious only a year or two ago. Although we are rapidly developing the tools to explore and understand secretion at the level of molecular interactions, the excitement for the future is really about what we do not know! Undoubtedly, the best we can expect is that the answers will be unexpected.

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