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INOSITOL LIPID METABOLISM AND SIGNAL TRANSDUCTION IN CLONAL PITUITARY CELLS

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SUMMARY

A number of clonal cell lines derived from a rat pituitary tumour, collectively termed GH cells, have retained a range of differentiated cell functions, including their ability to secrete the hormones prolactin and growth hormone in response to stimuli such as thyrotropin-releasing hormone (TRH). The mechanisms underlying this release process involve, at least in part, an increase in cytosolic free calcium levels, and the cells have proved useful as a model system in studies of receptor-controlled calcium mobilization. The initial response of the cells to the addition of TRH now appears to be the interaction of the occupied TRH receptor with a GTP-binding protein. A sophisticated signalling system is then activated which initially involves the phosphodiesteratic hydrolysis of phosphatidylinositol 4,5-bisphosphate to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. Both of these products are important intracellular messengers, and their formation leads to a plethora of biochemical and electrical changes which culminate in the biphasic release of hormone from the cell. The changes in cytosolic free calcium that occur following TRH addition follow a complex temporal pattern. Within 1 s, the concentration starts to increase from a resting level, in the range 100–150 nmol l^{-1}, to a peak value of around 1 μmol l^{-1} which is attained within 6–8 s. This 'spike' of calcium is almost exclusively derived from intracellular stores, probably the endoplasmic reticulum, in response to the formation of inositol 1,4,5-trisphosphate. With high concentrations of the peptide, the cytosolic free calcium concentration declines promptly, due to the activation of a protein kinase C-mediated extrusion and/or sequestration process. This inhibitory phase is less marked at low agonist concentrations but, in all cases, is superseded by a second increase in free calcium, which is due to the stimulated influx of the cation through dihydropyridine-sensitive calcium channels. These biphasic changes in calcium, in concert with the activation of protein kinase C, appear sufficient to regulate prolactin secretion.

INTRODUCTION

Studies of the biochemical mechanisms underlying hormone release from cells of the anterior pituitary gland are compromised by the diversity of cells found in most hemipituitary preparations and dispersed cell cultures and the lack of target cell specificity found with a number of the hormone-releasing and release-inhibiting factors. One alternative approach to the problem is to resort to the various clonal cell

Key words: inositol trisphosphate, diacylglycerol, thyrotropin-releasing hormone.

lines derived from anterior pituitary tumours. The most studied, in almost all respects, are GH_3 and related cell lines, which were derived by Tashjian *et al.* (1968) from an irradiation-induced, multihormone-producing rat tumour, termed MtT/W5. This family of cell lines produces, in general, both growth hormone (GH) and prolactin, the relative proportions of which depend on the particular cell line (Martin & Tashjian, 1977). The cells have a wide variety of features which are shared, as far as can be determined, by the corresponding normal rat pituitary cells, and indeed, in some instances, an action of pituitary hormone release modulators has been discovered using these model cells, and only subsequently been noted in the whole animal. Thus, the prolactin-releasing ability of the thyrotropin-releasing hormone (TRH), first detected on GH_3 cells (Tashjian, Barowsky & Jensen, 1971), was later shown to be present in a variety of animal species and, perhaps, to be of physiological relevance during suckling (see Leong, Frawley & Neill, 1983, for a review). It is also evident that there are differences between the tumour cell lines and their normal counterparts. For example, most of the cells currently used appear to lack dopamine receptors, which are, of course, involved in the physiological regulation of prolactin release. In addition, the cells' ability to store prolactin, at least under routine culture conditions, is much diminished by comparison with the normal cell. Recently, Dannies and her collaborators have provided evidence that this aspect of the tumour cell's performance can be corrected by the inclusion in the growth medium of a combination of various growth factors and hormones, indicating that the defect may derive not from the transformed nature of the cells, but from a deficiency in cell culture methodology (Scammel, Burrage & Dannies, 1985). With these caveats, then, the cells represent an extremely useful system for studying anterior pituitary hormone release.

One attraction of the cells to biologists, in general, and pharmacologists, in particular, is the wide variety of receptors which they exhibit both on their cell membrane and at intracellular loci (Table 1). The membrane receptors can either stimulate (TRH, bombesin, epidermal growth factor, vasoactive intestinal polypeptide, platelet-derived growth factor) or inhibit (acetylcholine and somatostatin) the release of prolactin (see Gershengorn, 1986, for a recent review), and for at least two of these receptors (TRH and bombesin) stimulated secretion depends, in part, on the mobilization of intracellular calcium (Gershengorn, 1986).

Our initial interest in these clonal cell lines derived from their possible use in studies relating agonist-induced calcium mobilization to the accompanying inositol lipid breakdown (Michell, 1975). The mobilization of intracellular calcium by receptor agonists is now recognized to depend crucially upon the stimulation, by the occupied receptor, of inositol lipid hydrolysis (see Berridge, this volume; Downes & Michell, 1985). This yields the water-soluble messenger molecule, inositol 1,4,5-trisphosphate (Ins $1,4,5P_3$), which can mobilize calcium from intracellular stores, probably from a portion of the endoplasmic reticulum in the vicinity of the plasma membrane (Berridge & Irvine, 1984). This transmembrane signalling mechanism is now identified as only a part of an increasingly complex system which is involved in controlling almost all aspects of the cell's performance, including ion channel

regulation. The elucidation of the extent of this system, and the details of how it is controlled in cells, are of relevance to all areas of cell biology. One way of facilitating this work is to use a simple model system such as the clonal GH cell lines, and this paper outlines the studies using these cells which bear on this problem.

POLYPHOSPHOINOSITIDE HYDROLYSIS IS THE INITIAL RESPONSE OF GH_3 CELLS TO THYROTROPIN-RELEASING HORMONE

As with most tissues, studies of inositol lipid metabolism in GH_3 cells commenced with a number of reports that an agonist, in this case TRH, could specifically

Table 1. *GH cell receptors*

Agonist	Kd ($nmol\,l^{-1}$)	No. of receptors/cell	Modulation of prolactin release	Cytosolic free calcium	Transduction pathway	Reference
Thyrotropin-releasing hormone (TRH)						
	10	135 000	++	++	Inositol lipid breakdown	a
Bombesin						
	1	3 600	+	+	Inositol lipid breakdown	b
Somatostatin						
	1	13 000	−	−	? Decrease cyclic AMP ?	c
Acetylcholine						
	1*	10 000	−	−	? Decrease cyclic AMP ?	d
Vasoactive intestinal polypeptide (VIP)						
	2	9 000	++	+	Increase cyclic AMP	e, h
Insulin						
	2	10 000	NE	−	? Tyrosine kinase ?	f, h
Epidermal growth factor (EGF)						
	1	34 000	+	±	? Tyrosine kinase ?	g, h, k
Platelet-derived growth factor (PDGF)						
	?	?	+	+	? Tyrosine kinase ?	h
Fibroblast growth factor (FGF)						
	?	?	+	+	?	i, j

* Measured as [^{3}H]QNB (quinuclidinylbenzilate) binding. NE, no effect; (+) increase; (−) decrease.
References: (a) Hinkle & Tashjian (1973); (b) Westendorf & Schonbrunn (1983); (c) Schonbrunn & Tashjian (1978); (d) Onali *et al.* (1983); (e) Bjoro *et al.* (1985); (f) Kiino & Dannies (1981); (g) Sullivan & Tashjian (1983); (h) Albert & Tashjian (1985); (i) Schonbrunn, Krasnoff, Westendorf & Tashjian (1980); (j) Wrench, Brown & Dobson (1985); (k) Johnson, Baxter, Vlodavsky & Gospodarowicz (1980).
This table is based, in part, on data collated by Dr P. M. Hinkle.

stimulate the incorporation of inorganic phosphorus into phosphatidic acid (PA) and phosphatidylinositol (PI) (Rebecchi, Monaco & Gershengorn, 1981; Drummond & Macphee, 1981; Schlegel, Roduit & Zahnd, 1981; Sutton & Martin, 1982). Since this method of studying the inositol lipid cycle was known to monitor the resynthesis of PI after stimulated inositol lipid hydrolysis and not the initial reactions of the cycle, attention soon shifted to these latter considerations. Studies using cultured cells are considerably facilitated relative to intact tissues because of the ease with which metabolites can be prelabelled to isotopic equilibrium using an appropriate precursor: a change in the radioactive labelling of the metabolite, under these conditions, reflects an alteration in the cellular mass of that substance. When this strategy was applied to GH_3 cells, it became obvious that TRH could elicit an increase in the cellular content of PA and 1,2-diacylglycerol (DAG) (Drummond & Macphee, 1981; Rebecchi, Kolesnick & Gershengorn, 1983; Martin, 1983; Macphee & Drummond, 1984) and a decrease in the content of PI (Rebecchi *et al.* 1983; Macphee & Drummond, 1984). The latter effect was entirely consistent with the view that PI hydrolysis by an agonist-catalysed activation of a phosphodiesterase (phospholipase C) was the initiating event in the activation of the cycle (Michell, 1975). However, studies of polyphosphoinositide metabolism in GH_3 and a variety of other cells have shown that there are more rapid early changes in both phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) following TRH addition (Rebecchi & Gershengorn, 1983; Martin, 1983; Macphee & Drummond, 1984; Schlegel *et al.* 1984*a*; and see Downes & Michell, 1985, for a recent review). An investigation of the production of the corresponding inositol phosphates in response to TRH has revealed that three phosphates (inositol tris-, bis- and monophosphate) accumulate within 10 s (Rebecchi & Gershengorn, 1983; Martin, 1983; Drummond, Bushfield & Macphee, 1984). In two of these reports (Rebecchi & Gershengorn, 1983; Martin, 1983), the production of inositol tris- and bisphosphate clearly preceded that of inositol monophosphate, indicating that PIP_2 and PIP were more likely substrates for phosphodiesterase action than PI itself. The third report (Drummond *et al.* 1984), in which the kinetics of the TRH effect appeared rather faster, demonstrated by using a lithium pre-treatment protocol and reduced temperature that the polyphosphoinositides, and in particular PIP_2, were the receptor-controlled substrates.

In the past 2 years, there has been an increasing emphasis on elucidating the mechanism by which the receptor is coupled to the phosphodiesteratic hydrolysis of the polyphosphoinositides. It is evident that there are a variety of soluble and plasma membrane-located phosphodiesterases which will attack inositol-containing lipids (see Downes & Michell, 1985). If the receptor-controlled enzyme is a soluble entity then it becomes necessary to invoke either an activating factor (which seems needlessly complex) or the possibility that it is a change in the accessibility of the substrate rather than an activation of the enzyme which controls the process (Irvine, Hemington & Dawson, 1979). If the receptor-controlled enzyme is plasma membrane-located, the link with the receptor becomes, in principle at least, much simpler. The latter situation also raised the possibility, first mooted by Gomperts

(1983) and actively pursued by Haslam & Davidson (1984*a*,*b*) that, as is the case with receptors which are coupled to adenylate cyclase, calcium-mobilizing receptors may be coupled to inositol lipid hydrolysis *via* a GTP-binding protein analogous to N_s or N_i. There is now rather good evidence that this possibility is the correct one, and the information that has led to this view can be summarized by reference to the appropriate GH cell data. The first hint that such a mechanism might exist came from the studies of Hinkle & Kinsella (1984), who showed that the binding of TRH to its receptor is inhibited by GTP and the non-hydrolysable GTP analogue guanylyl-5′-imidodiphosphate [Gpp(NH)p]. Subsequent work from the same laboratory indicated that TRH can stimulate the hydrolysis of GTP by a high-affinity GTPase (Hinkle & Phillips, 1984). Both of these properties are shared by receptors which are coupled to adenylate cyclase through a GTP-binding protein, although there is little or no evidence to suggest that the TRH receptor on GH cells is directly coupled to changes in cyclic AMP in this manner (Gershengorn, 1982). More recently, Lucas, Bajjalieh, Kowalchyk & Martin (1985) have shown that GTP and TRH can act synergistically to stimulate the hydrolysis of PIP_2 and PIP. Taken together with the data emerging from studies on other tissues (Haslam & Davidson, 1985; Cockcroft & Gomperts, 1985; Litosch, Wallis & Fain, 1985; Smith *et al.* 1985), it is evident that there is now a consensus in favour of a GTP-binding protein being involved in transduction through calcium-mobilizing receptors. Interestingly, although the data from GH_3 cells tend to indicate that neither N_s nor N_i is involved in this process (pertussis and cholera toxins are without effect; Lucas *et al.* 1985; Schlegel, Wuarin, Zbaren & Zahnd, 1985; Hinkle, Hewlett & Gerschengorn, 1986), there is reason to believe that in certain types of cell (e.g. neutrophils, mast cells) a pertussis toxin substrate plays a role (Okajima & Ui, 1984; Bokoch & Gilman, 1984; Nakamura & Ui, 1985). It remains to be determined whether one or, as seems more likely at present, a family of GTP-binding proteins are involved in the coupling of receptors to inositol lipid metabolism.

INOSITOL 1,4,5-TRISPHOSPHATE AND CALCIUM MOBILIZATION

There is now considerable evidence in favour of the hypothesis originally proposed by Berridge (1983) that inositol 1,4,5-trisphosphate (Ins 1,4,5P_3), produced in response to agonist-induced inositol lipid hydrolysis, subserves a calcium-mobilizing function in eukaryotic cells (see Berridge & Irvine, 1984, for a review). Using permeabilized GH_3 cells, Gershengorn and his collaborators (Gershengorn, Geras, Purrello & Rebecchi, 1984) have demonstrated that Ins 1,4,5P_3 can mobilize calcium from a non-mitochondrial pool, presumably a portion of the endoplasmic reticulum, as appears to be the case in other tissues (Streb *et al.* 1984). If Ins 1,4,5P_3 is indeed the physiological mediator of agonist-induced calcium mobilization, then it should be produced fast enough and in sufficient quantities to elicit calcium release and, as a consequence, alter the free cytosolic calcium concentration ($[Ca^{2+}]_i$). After TRH addition, there is a rapid 'spike' of InsP_3 production which is maximal within 5 s (Martin, 1983; Drummond *et al.* 1984). In our hands, at least, and measured under

identical conditions, this precedes the early 'spike' of $[Ca^{2+}]_i$), which peaks at around 6–8 s and is due almost entirely to the mobilization of intracellular calcium (Drummond, Knox & Macphee, 1985). The amount of Ins P_3 produced under those conditions can be calculated using the cellular content of PI, measured by inorganic phosphorus, as a reference if it is assumed that [^{3}H]inositol has equilibrated fully with its endogeneous counterpart during the 72-h labelling period. This indicates that around 20 μmol l^{-1} Ins P_3 is formed within 5 s of TRH addition to GH_3 cells (Drummond *et al.* 1984). This is significantly higher than the concentration of Ins 1,4,5P_3 which, in a variety of tissues, half-maximally elicits calcium mobilization (around 0·5 μmol l^{-1}; see Berridge & Irvine, 1984). There are reasons to believe, however, that only a proportion of the putative GH_3 cell Ins P_3 may be Ins 1,4,5P_3. Irvine and his colleagues (Irvine, Letcher, Lander & Downes, 1984; Irvine, Anggard, Letcher & Downes, 1985) have described the presence in parotid fragments of an atypical isomer Ins 1,3,4P_3, which is produced more slowly than Ins 1,4,5P_3 in response to agonist stimulation. A small amount of the stimulated Ins P_3 formation in GH_3 cells may be the 1,3,4-isomer. Also, the recent discovery that inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5 P_4) exists in rat brain and that its formation is stimulated by receptor agonists (Batty, Nahorski & Irvine, 1985) has alerted workers to the fact that 'Ins P_3' fractions eluted by high salt from ion-exchange columns may, in most cases, be contaminated by Ins 1,3,4,5P_4. This tetraphosphorylated inositol derivative also exists in GH_3 cells (Heslop, Irvine, Tashjian & Berridge, 1985) and its formation is stimulated by TRH (Heslop *et al.* 1985; L. A. Joels & A. H. Drummond, unpublished work). We estimate that as much as 60 % of the Ins P_3 fraction produced in GH_3 cells in response to TRH may be the tetrakisphosphate. At the time of writing there are no published data on the calcium-mobilizing ability of this agent, and for the purposes of this calculation we have ignored any such contribution. When these 'contaminating' metabolites are taken into account, the amount of Ins 1,4,5P_3 produced within 5 s of TRH addition falls to around 5 μmol l^{-1}. This is still rather higher than required to mobilize intracellular calcium in permeabilized cells, but is entirely consistent with the report that 10- to 30-fold higher concentrations of Ins P_3 are produced by maximal TRH concentrations than are necessary to produce maximal changes in $[Ca^{2+}]_i$ (Drummond *et al.* 1985). The concentrations of Ins 1,4,5P_3 which are produced by TRH concentrations around 1 nmol l^{-1}, where there is a direct coupling between Ins P_3 and calcium changes, will be sub-micromolar, exactly in the range predicted as necessary from the *in vitro* experiments.

A related point is the question of how much Ins 1,4,5P_3 exists in unstimulated GH_3 cells. Calculations of 'apparent' total Ins P_3 levels in resting GH_3 cells yield a value in excess of 5 μmol l^{-1}, rather more than is necessary to empty receptor-sensitive calcium pools in the cells. While as much as 80 % of this can probably be accounted for as Ins 1,3,4P_3 and Ins 1,3,4,5P_4, one is forced to the conclusion that, barring the presence of other impurities, Ins 1,4,5P_3 levels in unstimulated cells may be sufficiently high for the substance to be involved in the control of resting $[Ca^{2+}]_i$.

1,2-DIACYLGLYCEROL AND PROTEIN KINASE C ACTIVATION

Protein kinase C was first identified in 1977 as a proteolytically-activated protein kinase (Inoue, Kishimoto, Takai & Nishizuka, 1977). Subsequently, it was shown that, in the absence of protease action, the enzyme could be controlled by membranes, more specifically by acidic phospholipids such as phosphatidylserine, and by calcium ions (Takai *et al.* 1979). The link between this ubiquitous enzyme and signal transduction was provided by Nishizuka and his collaborators (see Nishizuka, 1984, for a recent review), who demonstrated that DAG (which, with Ins 1,4,5P_3, is the primary product of PIP_2 hydrolysis) increases the affinity of protein kinase C for calcium and, as a result, leads to its activation. From these observations grew the idea that inositol lipid hydrolysis could provide the cell with a bifurcating signalling system in which calcium and protein kinase C, acting in tandem, might control a range of cellular processes (see Nishizuka, 1984; Berridge, 1984).

Addition of TRH to GH_3 cells leads to the rapid phosphorylation of a variety of cytosolic proteins (Drust & Martin, 1982; Drust, Sutton & Martin, 1982; Sobel & Tashjian, 1983). Drust & Martin (1984) have reported that of six proteins phosphorylated during TRH stimulation of GH_3 cells, five are substrates for protein kinase C while the other, a 97 K protein, is phosphorylated in a calcium-dependent manner, presumably by a Ca^{2+}/calmodulin-dependent protein kinase. This analysis is based on the various pharmacological tools which are available for studying the role of protein kinase C in a physiological process, namely the ability of protein kinase C-activators such as the tumour-promoting phorbol esters, membrane-permeable diacylglycerols and bacterial phospholipase C to mimic the response of the agonist. To date, none of these proteins have an allocated function in the cellular events activated by TRH. It is important to emphasize the speed with which the steps leading to this protein kinase C-dependent phosphorylation occur. DAG is produced within 5–10 s of TRH addition (and probably much faster) (Rebecchi *et al.* 1983; Martin, 1983; Macphee & Drummond, 1984). Recently, two groups have demonstrated that this, in turn, leads to the translocation within 15 s of protein kinase C from the cytosol to the plasma membrane (Drust & Martin, 1985; Fearon & Tashjian, 1985), where presumably it is activated by the combined action of DAG, calcium and phosphatidylserine. Such a time frame is entirely commensurate with the phosphorylation of certain of these cytosolic proteins, the phosphorylation of which can be detected 10–15 s after TRH addition (Drust & Martin, 1984).

One area of growing interest is the question of how, and with what time course, this process is terminated. Translocation of the enzyme to the membrane is an ephemeral phenomenon: within 2 min of TRH addition, the specific activity of protein kinase C in the particulate fraction of the cell has returned towards unstimulated levels (Drust & Martin, 1985; Fearon & Tashjian, 1985), although there may be a small persistent pool remaining in the membrane (Drust & Martin, 1985). This latter observation is in accord with the reports that DAG levels in TRH-stimulated GH_3 cells remain elevated for a considerable time after addition of the agonist (Martin, 1983; Macphee & Drummond, 1984; Drummond & Raeburn,

1984). Because much of the protein kinase C returns to the cytosol despite the continued presence of DAG in the cell membrane, it is necessary to postulate the existence of other mechanisms controlling the distribution of the enzyme. Of relevance here is the finding that there appears to be a significant net loss of enzyme activity during the cycling of the enzyme to and from the membrane (Drust & Martin, 1985; Fearon & Tashjian, 1985), and a similar result is obtained when the enzyme is measured by its phorbol ester-binding ability (Fearon & Tashjian, 1985). Membrane-associated protein kinase C is particularly susceptible to calcium-activated proteolysis, and this leads to the formation of a 51 K fragment of the enzyme which, devoid of its hydrophobic region, operates independently of calcium, DAG and acidic phospholipids (Kishimoto, Kajekawa, Shiota & Nishizuka, 1983). It remains to be seen whether such a process operates in GH_3 cells and, if so, whether it constitutes a mechanism both for terminating membrane protein phosphorylation, related perhaps to the regulation of the early events in the stimulated cell, and for allowing the enzyme access to intracellular targets that might be important in the control of longer-term functions such as hormone synthesis or replicative phenomena.

The role of protein kinase C in controlling cellular calcium levels and in the release of prolactin from GH_3 cells is discussed in the next three sections.

CONTROL OF THE CYTOSOLIC FREE CALCIUM CONCENTRATION IN GH CELLS

The calcium signal resulting from the addition of TRH to GH_3 cells and related clones is complex. It appears to depend both on the activation of the two limbs of the inositol lipid signalling system, which is the crucial first step, and upon the influx of calcium from the extracellular *milieu* through voltage-sensitive calcium channels. A number of studies have demonstrated that the increase is biphasic: there is a rapid, large elevation in $[Ca^{2+}]_i$ which peaks within 6–8 s, returns rapidly towards control values, and is followed by a prolonged 'plateau' phase in which the $[Ca^{2+}]_i$ is moderately increased (Gershengorn & Thaw, 1983; Albert & Tashjian, 1984*a*; Schlegel & Wollheim, 1984). The evidence suggests that most, if not all, of the first phase of the calcium signal derives from intracellular sources (Gershengorn & Thaw, 1983; Albert & Tashjian, 1984*a*; Schlegel & Wollheim, 1984; Snowdowne & Borle, 1984). First, removal of extracellular calcium shortly before addition of the peptide has little effect on the initial 'spike' of calcium, whilst preventing the subsequent 'plateau' phase. Secondly, the first phase is largely resistant to the presence of calcium channel blockers such as nifedipine and verapamil, which markedly reduce the second phase (Albert & Tashjian, 1984*b*; Gershengorn & Thaw, 1985). As discussed above, there is substantial evidence that Ins 1,4,5P_3 plays a role in producing the initial calcium 'spike'.

As the concentration of TRH agonist is increased, it is evident that the calcium 'spike', while larger, becomes increasingly short-lived (Drummond *et al.* 1985). Recently, it has been suggested that this is due to the fact that TRH, in addition to stimulating calcium mobilization due to Ins 1,4,5P_3 formation, may also activate

an inhibitory process which leads to the rapid reversal of the calcium signal (Drummond, 1985). When $[Ca^{2+}]_i$ is artificially elevated due to the opening of voltage-dependent calcium channels by high concentrations of K^+, the process which facilitates the reversal of the initial calcium 'spike' becomes much more obvious (Drummond, 1985). In GH_4C_1 cells, the resting $[Ca^{2+}]_i$ is already high (300 nmol l^{-1} *versus* approximately 150 nmol l^{-1} in most GH_3 cell lines) and in this clone TRH can, after the initial calcium 'spike', reduce $[Ca^{2+}]_i$ below the unstimulated value (Albert & Tashjian, 1984*b*, 1985). This presumably is a manifestation of the same process. It seems likely that this inhibitory effect of TRH is mediated by protein kinase C and, therefore, that both limbs of the inositol lipid signalling system converge to control the early changes in $[Ca^{2+}]_i$ (Drummond, 1985). The evidence supporting this suggestion is outlined below. First, the inhibitory effect of TRH is mimicked by phorbol esters which are known to activate protein kinase C, such as phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-didecanoate (PDD), but not by those which are not, such as 4-α-phorbol and 4-α-phorbol 12,13-didecanoate (Drummond, 1985; Albert & Tashjian, 1985). Secondly, bacterial phospholipase C, which stimulates a large increase in the GH_3 cell DAG content, leads to the translocation and activation of protein kinase C and, at least when low activities of the enzyme are used, does not increase Ins P_3 production (Drust & Martin, 1984, 1985; Drummond, 1985), also elicits an inhibitory effect on $[Ca^{2+}]_i$ when added after high concentrations of K^+ (Drummond, 1985). Thirdly, at the time when the TRH-induced inhibitory effect becomes manifest (approximately 10 s after peptide addition) DAG levels have been elevated, protein kinase C has been translocated and phosphorylation of the phorbol ester-sensitive proteins has been effected (Rebecchi *et al.* 1983; Martin, 1983; Macphee & Drummond, 1984; Drust & Martin, 1984, 1985). The mechanisms which might underlie this protein kinase C-mediated inhibitory action are discussed in the next section.

The 'plateau' phase of the calcium signal, which follows these two mutually opposing actions of TRH, appears to be entirely dependent on the influx of extracellular calcium. As mentioned above, it does not occur in the absence of extracellular calcium and is markedly reduced in the presence of drugs such as nifedipine or verapamil which are known to block the entry of calcium through voltage-dependent channels. It is important to emphasize, however, that although it ultimately depends on changes in the electrical activity of the GH_3 cell, the genesis of these changes can probably be ascribed to the initial stimulation of inositol lipid metabolism. For example, the various facets of the calcium signal can be reproduced exactly by the addition of a combination of the calcium ionophore ionomycin and PMA (Albert & Tashjian, 1985).

In understanding the second or 'plateau' phase of the calcium signal, it is important to discuss the electrophysiological and ion-flux studies which have contributed to our understanding of GH cell biology. Since, due to the limitations of space, the coverage is necessarily selective, the interested reader is referred to the review by Tixier-Vidal, Tougard, Dufy & Vincent (1982) for a more thorough treatment.

A substantial, but variable, number of GH cells in a population exhibit spontaneous electrical activity (see Tixier-Vidal *et al.* 1982), and it is evident from a variety of studies that alterations in both Na^+ and Ca^{2+} currents underlie these action potentials (Kidokoro, 1975; Biales, Dichter & Tischler, 1977; Ozawa & Miyazaki, 1979). Addition of TRH to GH cells has been shown both to enhance the frequency of spontaneous action potentials in active cells, and also to induce 'spiking' in quiescent cells (Kidokoro, 1975; Dufy *et al.* 1979; Ozawa & Kimura, 1979; Taraskevich & Douglas, 1980). The response to TRH is, however, rather complex with the initial response to the peptide being not an activation, but an inhibition of action potential firing (Ozawa & Kimura, 1979; Ozawa, 1981; Dufy, Israel, Zyzek & Gourdji, 1982). This is associated with membrane hyperpolarization and an increase in membrane conductance (Ozawa & Kimura, 1979). This hyperpolarization has a latency of around 1 s, lasts for as much as 30 s and is believed to be mediated by the activation of $I_K(Ca)$ (Ozawa, 1981, 1985; Dubinsky & Oxford, 1985). Since TRH does not alter single, Ca^{2+}-activated K^+-channel behaviour in excised membrane patches (Dubinsky & Oxford, 1985), it seems likely that the opening of this channel is mediated by the release of calcium, from intracellular stores, presumably by the Ins 1,4,5P_3-dependent process discussed above. In some clones, such as GH_4C_1 cells, in which it is apparent that the activation of voltage-dependent calcium channels contributes significantly to the basal $[Ca^{2+}]_i$, the hyperpolarization mediated by the opening of $I_K(Ca)$ may contribute to the rapid cessation of the initial calcium 'spike' (Albert & Tashjian, 1984*b*, 1985). After the hyperpolarizing phase of the TRH response has waned, there is a marked increase in action potential frequency (Kidokoro, 1975; Dufy *et al.* 1979; Ozawa & Kimura, 1979). This persists in Na^+-free medium, and is probably largely due to the enhanced opening of voltage-sensitive calcium channels (Ozawa & Kimura, 1979). There is, however, no evidence that TRH can directly open calcium channels in GH cells: cells internally-dialysed with K^+-free solutions show no change in calcium currents in response to TRH (Dubinsky & Oxford, 1985). Indeed, preliminary experiments with PMA or purified protein kinase C appear to indicate that these excitatory electrical changes may be secondary to the activation of this enzyme (O. Sand & B. Dufy, personal communications). The explanation for the anomaly reported above appears to reside in the fact that TRH can alter the activity of another K^+ current, $I_K(V)$. Voltage-clamp studies indicate that the rate of onset and amplitude of this voltage-activated K^+ current are decreased by TRH (Dubinsky & Oxford, 1985). Because of the depression of this current, the probability that voltage-sensitive calcium channels will open is enhanced due to the increased fraction of time which the cell membrane will spend in a depolarized state (Barros *et al.* 1985; Dubinsky & Oxford, 1985). The nature of the calcium channels which open in response to TRH has not yet been reported; there is evidence from whole-cell patch-clamp studies that GH_3 cells contain two distinct types of calcium channels which can be distinguished on the basis of their closing kinetics (Armstrong & Matteson, 1985). It is clear, however, that they are sensitive to the range of pharmacological agents which have been shown to affect conductance through calcium channels in other electrically excitable tissues.

Thus, organic substances such as nifedipine and its derivatives, verapamil and diltiazem inhibit ion flux through these channels (Tan & Tashjian, 1984*a*,*b*; Albert & Tashjian, 1984*b*; Enyeart, Aizawa & Hinkle, 1985), whilst activators such as BAYK8644, CGP 28392 and maitotoxin stimulate calcium entry (Enyeart & Hinkle, 1984; Shangold, Kongsmaut & Miller, 1985; Login *et al.* 1985). As is found in other tissues, calcium entry stimulated by depolarization with high K^+ is more sensitive to pharmacological blockade than is agonist-induced influx (Enyeart *et al.* 1985).

It has been suggested that the influx of calcium through nifedipine- or verapamil-sensitive calcium channels is only one of the features that contribute to the 'plateau' phase of the calcium signal (Albert & Tashjian, 1984*b*; Gershengorn & Thaw, 1985). Only around 50 % of the calcium influx mediating this phase can be accounted for by entry through these channels (Tan & Tashjian, 1984*b*). The mechanism(s) by which the remainder of the calcium gains entry into GH cells is unknown at the present time. One possibility might be uptake by means of Na^+/Ca^{2+} exchange. As mentioned above, there is significant entry of Na^+ during GH cell action potentials (Biales *et al.* 1977). GH cells are known to contain the Na^+/Ca^{2+} exchanger (Kaczorowski *et al.* 1984; Kaczorowski, Barros, Dethmers & Trumble, 1985), and it seems possible that some of the removal of this sodium from the cytosol may occur in exchange for extracellular calcium. Studies with the various amiloride analogues which have been identified as potent inhibitors of this exchange process (Kaczoroswki *et al.* 1985) will be interesting in this respect.

CONTROL OF RECEPTOR-STIMULATED CHANGES IN INOSITOL LIPID METABOLISM AND CALCIUM MOBILIZATION

It is becoming increasingly evident that receptor-stimulated calcium and inositol lipid changes are tightly controlled processes. This is, perhaps, not surprising given the central role which is now ascribed to these cellular phenomena. Moreover, one of the advantages that accrues from the cell's possession of a sophisticated signalling system such as this, as opposed to a more straightforward process such as the cyclic AMP-generating mechanism, is that the possibilities of both internal and external control are enormously increased. Thus, while we should expect that the regulatory mechanisms that govern receptor-signalling through adenylate cyclase/ cyclic AMP might also apply to inositol lipid-dependent signalling, it should not be a surprise if other novel control steps are discovered to be present.

At the present time, this aspect of the subject remains relatively unexplored. Some features are known, however. As with the cyclic AMP-generating system, there is an element of control at the level of the receptor. The number of TRH receptors on GH cells, for example, is controlled by a variety of processes. It is increased by certain hormones, such as glucocorticoids and oestrogens (Tashjian, Osborne, Maina & Knaian, 1977; Brunet, Gourdji & Tixier-Vidal, 1980), and decreased by others, such as the thyroid hormones (Perrone & Hinkle, 1978) and, of course, by TRH itself (Hinkle & Tashjian, 1975). Other messenger systems can also regulate TRH

receptor number. Recently, Imai & Gershengorn (1985) have demonstrated that agents which increase the GH_3 cell content of cyclic AMP can, after relatively long periods of incubation, down-regulate TRH receptors. It should be emphasized that these effects on TRH receptor number do not reflect generalized changes in membrane receptor proteins; other receptor types are unaffected by the above treatments. Of greater relevance in terms of the endogenous control of long-term signalling through the inositol lipid pathway is the report that the activators of protein kinase C, such as PMA, can decrease TRH receptor number on GH cells (Jaken, Tashjian & Blumberg, 1981*a*). Interestingly, the converse is also true: treatment of GH cells with TRH reduces the number of phorbol ester binding sites (Jaken *et al.* 1981*b*; Jaken, Feldman, Blumberg & Tashjian, 1983). Whether the former mechanism is employed by TRH in reducing its own receptor number awaits further clarification.

The existence of potential control steps beyond the receptor stage has also been reported. Short-term treatment of GH cells with agonists such as carbachol and somatostatin has been reported to decrease the size of the initial 'spike' of calcium in response to TRH (Schlegel, Wuarin, Wollheim & Zahnd, 1984*b*; Schlegel *et al.* 1985). The mechanism underlying this action remains elusive, although it may be important that, at the time of the inhibition, the cells are hyperpolarized (as judged by bis-oxonol fluorescence). Neither somatostatin nor carbachol affect TRH-induced $Ins P_3$ formation (C. H. Macphee & A. H. Drummond, unpublished work), although both agonists decrease the basal $[Ca^{2+}]_i$ (Schlegel *et al.* 1984*b*, 1985; Koch, Dorflinger & Schonbrunn, 1985).

Protein kinase C may be especially important as a regulator of receptor-stimulated inositol lipid metabolism and calcium mobilization (Drummond & MacIntyre, 1985). As indicated above, there is substantial evidence to suggest that TRH and more direct activators of protein kinase C, such as PMA, can exert inhibitory actions after the calcium signal has been elicited. The mechanisms underlying these effects have not yet been properly investigated, but it is apparent that the enzyme can affect many of the steps in the inositol lipid cycle and a range of the cellular processes that regulate $[Ca^{2+}]_i$. Both the basal and TRH-stimulated turnover of inositol lipids are decreased by preincubation with a variety of active phorbol esters (Drummond, 1985; Fig. 1). The inhibition of $Ins P_3$ production is particularly marked when a low concentration of the stimulant is used (Fig. 2). These data are similar to those that have been reported in a number of tissues (see, for example, MacIntyre, McNicol & Drummond, 1985; Vicentini *et al.* 1985). It remains unclear whether these effects are mediated by a direct effect on the PIP_2 phosphodiesterase, as seems possible from the fact that the basal turnover is affected, or are manifestations of the effective uncoupling of the receptor from the enzyme. Moreover, despite the widespread nature of these phenomena, their physiological relevance will remain in doubt until specific protein kinase C antagonists become widely available.

While it is unlikely that the rapid reversal of the calcium signal observed with high concentrations of TRH is due to this effect of protein kinase C, it seems clear that the

enzyme plays some role in this process (Drummond, 1985). It is unlikely to be due to a direct effect of protein kinase C on voltage-sensitive calcium channels, (a) since their role in the early 'spike' of calcium is small, (b) because the addition of PMA prior to the addition of high K^+ does not appreciably affect the rate of rise of the resultant calcium signal in GH_3 cells (A. H. Drummond, unpublished work) and (c) because addition of PMA after pre-elevation of $[Ca^{2+}]_i$ with ionomycin, which does not open these channels, is also sensitive to inhibition by PMA and TRH (A. H. Drummond, unpublished work). Moreover, initial electrophysiological studies on GH_3 cells using PMA indicate that it stimulates, rather than inhibits, the opening of these channels (O. Sand, personal communication). The most likely mechanism for the inhibition of the calcium signal is that the extrusion or sequestration of the cation is altered by protein kinase C: there is evidence from other tissues that these steps can be affected by the enzyme (Limas, 1980; Lagast, Pozzan, Waldvogel & Lew, 1984). The widespread interference by protein kinase C in the processes leading to the controlling calcium mobilization are summarized in Fig. 3. It should be noted, however, that in certain tissues the enzyme can act as a positive regulator, for

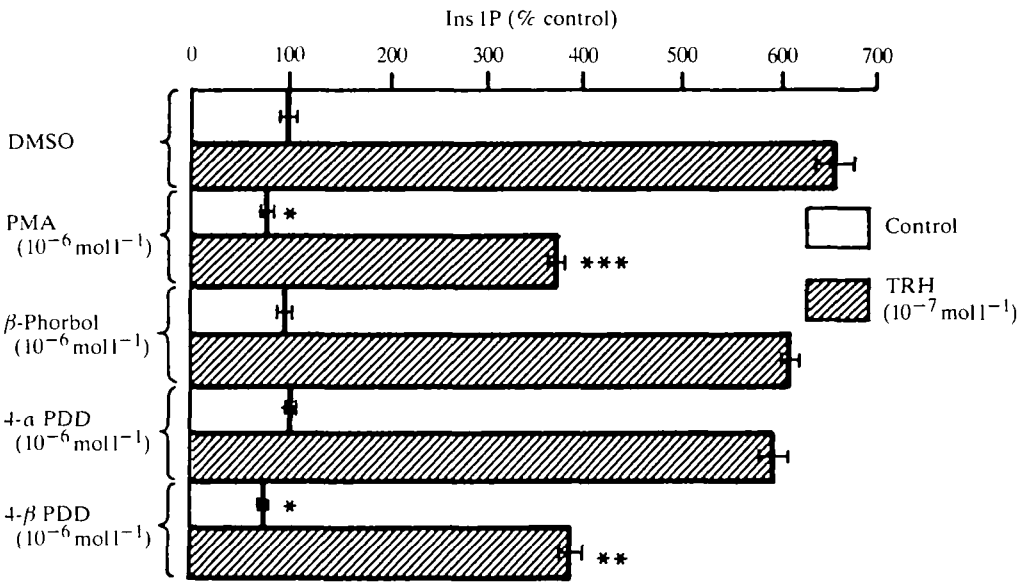

Fig. 1. GH_3 cells, which had been labelled to isotopic steady-state with [^{3}H]inositol for 48 h, were suspended in a balanced salt solution (Rebecchi, Monaco & Gerschengorn, 1981) in which 10 mmol l^{-1} of the constituent NaCl had been replaced by an equimolar amount of LiCl. After exposure to the appropriate phorbol derivative for 5 min at 37°C, samples of cell suspension were incubated with or without thyrotropin-releasing hormone (TRH) for a further 10 min. The reaction was then stopped and the lithium-induced accumulation of [^{3}H]Ins1P was measured by ion-exchange chromatography (Drummond, 1985). The open bars represent samples which were not exposed to TRH, while the filled bars represent samples which were exposed to TRH (10^{-7} mol l^{-1}). Values shown are means ± S.E.M. DMSO, dimethyl sulphoxide; PMA, phorbol 12-myristate 13-acetate; PDD, phorbol 12,13-didecanoate.

example, of Ins P_3 production in thymocytes (Taylor *et al.* 1984) and, indeed, other effects such as the stimulation of polyphosphoinositide synthesis (de Chaffoy de Courcelles, Roevens & Van Belle, 1984) and phosphatidate phosphohydrolase (Hall, Taylor & Saggerson, 1985) (Fig. 3) would also represent positive feedback effects.

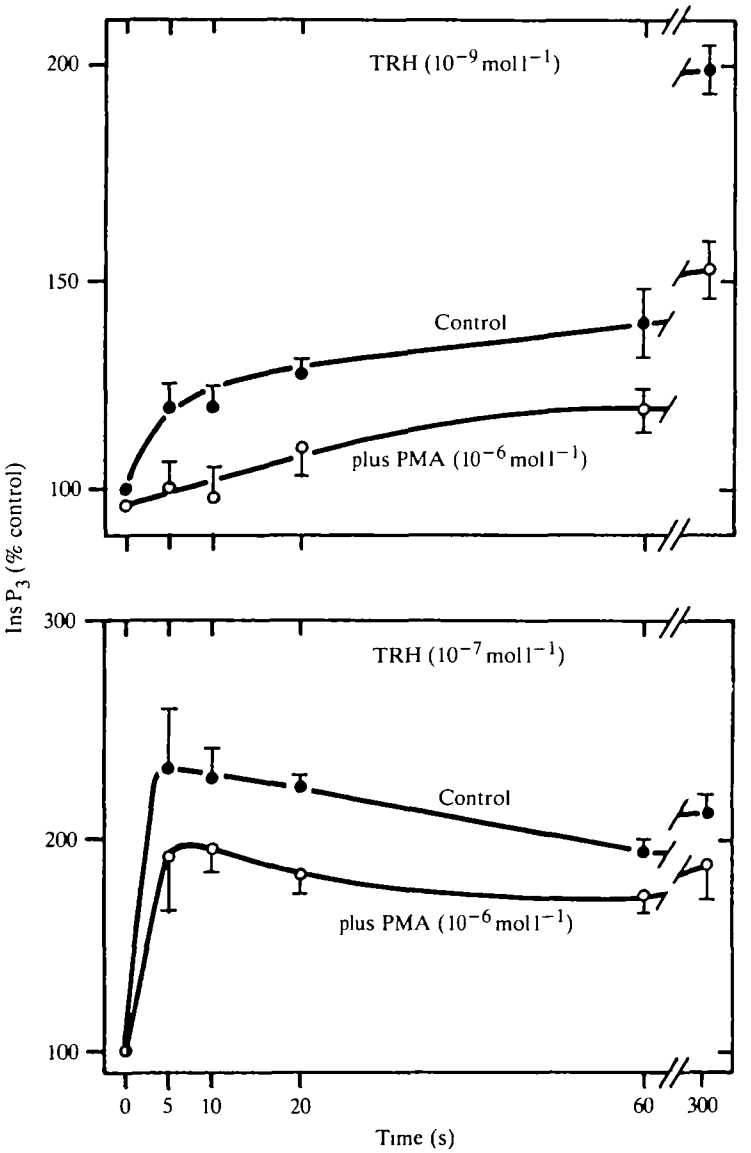

Fig. 2. GH_3 cells, labelled and prepared as described in the legend to Fig. 1, were preincubated with phorbol 12-myristate 13-acetate (PMA, 10^{-6} mol l^{-1}) for 5 min at 37°C prior to the addition of thyrotropin-releasing hormone (TRH) (10^{-9} mol l^{-1}; top panel) or TRH (10^{-7} mol l^{-1}; bottom panel) for the times shown. Reactions were terminated and [^{3}H]Ins P_3 fractions isolated as described previously (Drummond, 1985). The values shown are means ± S.E.M.

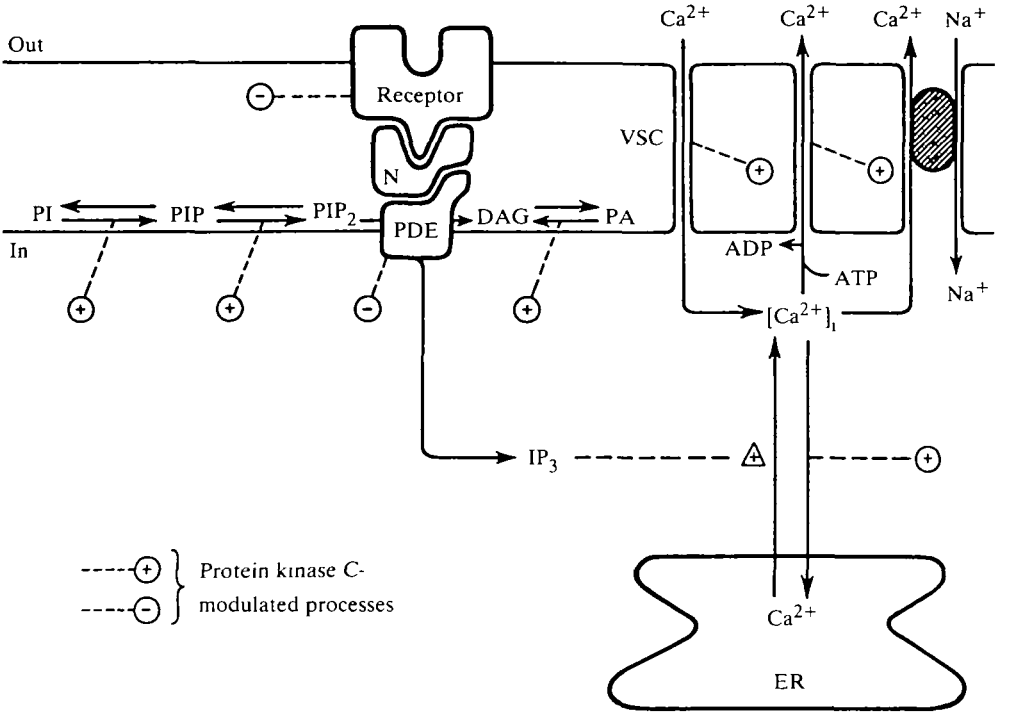

Fig. 3. A schematic representation of the processes which regulate cytosolic free calcium in mammalian cells and their control by protein kinase C. Note that all of these mechanisms have not necessarily been shown to apply in GH cells. VSC, voltage-sensitive calcium channel; PDE, phosphodiesterase; DAG, 1,2-diacylglycerol; PA, phosphatidic acid; N, GTP-binding protein; ER, endoplasmic reticulum.

Lastly, if protein kinase C can exert a multitude of effects on these processes, it seems equally probable that calcium will, either itself or in combination with protein kinase C, subserve similar control functions.

THE ROLE OF THE INOSITOL LIPID PATHWAY IN THE CONTROL OF PROLACTIN RELEASE

Although the calcium-dependence of TRH-stimulated prolactin release has been known for almost a decade (Tashjian, Lomedico & Maina, 1978), it is only within the last few years that significant advances have been made in understanding how the release process is controlled (see Gershengorn, 1986). The role of extra- *versus* intracellular calcium in the regulation of prolactin release has been a controversial area (see Gershengorn, 1982, 1986). Some workers, notably Gershengorn and his collaborators, have suggested that TRH-stimulated prolactin release is dependent only on the mobilization of intracellular calcium (Gershengorn, 1982). Others, using rather different experimental protocols, have noted that calcium influx is necessary (Tashjian *et al.* 1978; Ostlund *et al.* 1978). The identification of two experimental variables, most notably by Martin & Kowalchyk, 1984*b*), has helped to resolve the

controversy. First, differences existed in the way in which secretion was measured: Gershengorn's group used column-perfused GH cells, which will tend to emphasize rates of prolactin secretion, while the other two groups used relatively long and static incubations with the peptide, which tend to emphasize accumulation of the hormone. Secondly, Martin & Kowalchyk (1984*b*) demonstrated that the interpretation of data derived from experiments in which extracellular calcium had been chelated by EGTA is difficult owing to the speed with which the chelator strips the cells of intracellular calcium. In large part, the differences between the two camps have been resolved by the finding that the addition of TRH to GH cells elicits a biphasic release of prolactin: during the first 2 min after TRH addition (phase I) secretion of prolactin is rapid and markedly enhanced. Subsequently, a more sustained, but less marked, secretion occurs which persists for at least 60 min (phase II) (Martin & Kowalchyk, 1984*a*,*b*; Albert & Tashjian, 1984*a*,*b*; Delbeke, Kojima, Dannies & Rasmussen, 1984). The divergent results mentioned above probably result from the use of experimental conditions which select one or other phase of release. It seems likely that the sustained phase of secretion starts immediately after TRH addition, but only becomes visible after the 'burst' phase has waned (Kolesnick & Gershengorn, 1985). The two phases of secretion can be distinguished on the basis of their calcium dependence and the source of their calcium. Phase I appears to be entirely dependent on calcium mobilization from intracellular stores, while phase II depends, at least to some extent, on calcium influx (Martin & Kowalchyk, 1984*a*,*b*; Albert & Tashjian, 1984*a*,*b*). These properties are reminiscent of the two phases of TRH-stimulated $[Ca^{2+}]_i$ elevation mentioned above and, indeed, there is a good temporal correlation between these changes and the biphasic enhancement of secretion (Albert & Tashjian, 1984*b*). It is evident, however, that while phase I of prolactin release is largely mimicked by the addition of a calcium ionophore or high K^+ (see Albert & Tashjian, 1984*b*, for a dissenting report), phase II is not (Martin & Kowalchyk, 1984*a*,*b*; Delbeke *et al.* 1984). This latter secretory process appears to be more critically dependent on the activation of protein kinase C (Martin & Kowalchyk, 1984*a*; Delbeke *et al.* 1984; Albert & Tashjian, 1984*b*). The differentiation of the two phases of secretion into a Ca^{2+}-dependent phase and a protein kinase C-dependent phase is probably not absolute, however, and a careful assessment of the role of protein kinase C in phase I and calcium in phase II remains to be carried out. It seems likely that the basis for, at least part of, the remaining controversy regarding secretory mechanisms is the variability between the different GH cell clones in use.

While the central role of changes in inositol lipid metabolism, $[Ca^{2+}]_i$ and protein kinase C in the regulation of prolactin secretion is now widely established, the detailed steps that follow the second messenger changes remain unknown. Future work on this aspect will undoubtedly concentrate on the identification of the various proteins that are phosphorylated by protein kinase C and calcium/calmodulin-dependent protein kinase(s), their subcellular distribution and role in initiating and controlling the fusion of the prolactin-containing granules with the plasma membrane.

CONCLUSIONS

The versatility of GH_3 and related cell clones in terms of their utility as model cells is remarkable, and advocates of their use can be found in all disciplines of cell and molecular biology. The growth of knowledge regarding their inositol lipid metabolism, in particular, is a testament to this: in 5 years they have developed from a cell in which little or nothing was known of this subject, to a system which is amongst the best researched. Further developments will undoubtedly follow. Paramount amongst these, in my opinion, and the result of a timely liaison between biochemists, pharmacologists and electrophysiologists, will be the elucidation of the detailed mechanisms by which receptors regulate ion channel opening.

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