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The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells: defined characteristics and unanswered questions

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It now appears to be generally agreed that the 'phosphatidylinositol response', discovered in 1953 by Hokin & Hokin, occurs universally when cells are stimulated by ligands that cause an elevation of the ionized calcium concentration of the cytosol. The initiating reaction is almost certainly hydrolysis of an inositol lipid by a phosphodiesterase. Phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate all break down rapidly under such circumstances. However, we do not yet know which of these individual reactions is most closely coupled to receptor stimulation, nor do we know where in the cell it occurs. With many stimuli, inositol phospholipid breakdown is closely coupled to occupation of receptors and appears not to be a response to changes in cytosol [Ca^{2+}]: this provoked the suggestion that it may be a reaction essential to the coupling between activation of receptors and the mobilization of Ca^{2+} within the cell. In a few situations, however, it appears probable that inositol lipid breakdown can occur as a result of the rise in cytosol [Ca^{2+}] that follows receptor activation: such observations gave rise to the alternative opinion that inositol lipid breakdown cannot be related to stimulus–response coupling at calcium-mobilizing receptors. It now seems likely that these two views are too rigidly polarized and that some cells probably display both receptor-linked and Ca^{2+}-controlled breakdown of inositol lipids. Both may sometimes occur simultaneously or sequentially in the same cell.

Historical introduction

Almost thirty years ago, Hokin & Hokin (1953) observed an increase in membrane phospholipid turnover in exocrine pancreas that was stimulated to secrete amylase either by pancreozymin or by acetylcholine. Later, mainly during the 1950s and early 1960s, they also played the major roles in defining the general character of this response as a selective increase in the turnover of phosphatidylinositol (PtdIns), a relatively minor anionic membrane lipid. They found that this type of change in lipid metabolism is a widespread response of stimulated secretory tissues, both peripheral and neural (L. E. Hokin 1968, 1969). However, it was clear by 1968 that this response was not, as had originally been suspected, an essential component of the dynamic membrane events that are intrinsic to exocytotic secretion: it could be dissociated from exocytosis in some secretory cells and it also occurred very rapidly in non-secretory cells such as T lymphocytes (M. R. Hokin 1968*a*, *b*; Fisher & Mueller 1968, 1971). Ten years ago, therefore, enhancement of PtdIns metabolism by hormones, neurotransmitters and other ligands was known as a widespread, but still enigmatic, response of cells to stimulation.

The early 1970s saw the formulation of several conclusions that seemed likely to facilitate understanding of the mechanism and function of this type of response. The response seemed only to occur in cells stimulated by ligands that acted at cell-surface receptors (Lapetina & Michell 1973). Although usually detected as an increase in the labelling with ^{32}P of phosphatidate and PtdIns, the initiating biochemical event appeared always to be PtdIns breakdown. This had been observed in avian salt gland in 1964 (Hokin & Hokin 1964), but it was not realized that this observation could be generalized until much later (Durell *et al.* 1969; Hokin-Neaverson 1974*a*, *b*, 1977; Jones & Michell 1974; Michell 1975; Michell *et al.* 1977; Jones *et al.* 1979). Ten years ago, the emphasis in biochemical studies of the mechanisms involved in cell responsiveness to stimuli was on cyclic nucleotides, with only a few biochemists (e.g. Rasmussen 1970) joining the pharmacologists (e.g. Douglas 1968; Triggle 1972; Hurwitz & Suria 1971; Rubin 1974) who already recognized the central importance to cellular control of receptor-controlled changes in cytosol [Ca^{2+}]. As a result, the first realization that the multifarious cell-surface receptors that stimulated PtdIns metabolism also possessed another shared characteristic arose from their ability to elevate tissue concentrations of cyclic GMP (table VII of Michell 1975). At that time, however, a rise in cytosol [Ca^{2+}] was being implicated in the stimulation by receptors of cyclic GMP accumulation in cells (Berridge 1975; Goldberg & Haddox 1977). An obvious inference was that stimulated PtdIns turnover might be either involved in or a result of the receptor-stimulated elevation of cytosol [Ca^{2+}] (Michell 1975).

The more interesting of these two alternatives was that stimulated PtdIns turnover might involve an enzymic reaction that was essential to the chain of events through which activation of receptors elevated cytosol [Ca^{2+}]. However, there were three earlier observations which, taken together, had appeared to contradict the notion that the PtdIns response might be implicated in receptor–response coupling: most of the stimulated PtdIns labelling followed, rather than preceded, physiological responses (M. R. Hokin 1968*b*; Trifaró 1969*a*; Oron *et al.* 1975), physiological responses could be evoked fully by concentrations of agonist that evoked only a small proportion of the maximum PtdIns response (Hokin 1968*a*), and stimulated PtdIns metabolism, unlike other responses of the stimulated tissues, did not seem to require a supply of extracellular Ca^{2+} (Hokin 1966; Trifaró 1969*b*; Oron *et al.* 1975). But closer examination of these apparent objections showed them to be invalid. First, we have no detailed understanding of the precise relation between stimulated PtdIns labelling and inositol lipid breakdown, the primary event, so it is impossible to make valid assessments of the temporal or other kinetic characteristics of the PtdIns response by using such measurements of labelling. This problem was clearly stated many years ago (Michell 1975), but it is still not uncommon for inappropriate lipid labelling studies to form the basis for potentially important conclusions about the mechanism or function of PtdIns breakdown. To avoid confusion, it is essential that future studies concentrate wherever possible on measurements of inositol lipid breakdown (Michell & Kirk 1981*a*). Secondly, with the pharmacological demonstration of 'spare receptors', it was established that a full physiological response of a tissue to a ligand is often exhibited in response to activation of only a small fraction of the relevant receptor population. Under such conditions, any reaction essential to receptor–response coupling (e.g. activation or inhibition of adenylate cyclase, or possibly PtdIns breakdown) may only be submaximally activated (Michell *et al.* 1976*a*, *b*). Thirdly, a reaction involved in a coupling sequence that links activated receptors to the mobilization of Ca^{2+} need not itself require Ca^{2+} (Michell 1975). However, there is no reason why it should not, provided that the required amount of Ca^{2+} is available even in the unstimulated cell (i.e. a requirement in the submicromolar range).

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Far from rebutting the idea that inositol lipid breakdown might be involved in receptor–response coupling, it therefore appeared that its 'anomalous' dose–response curve and lack of a rigorous Ca^{2+} requirement might well presage such a role. We therefore developed as a working hypothesis the postulate, depicted in figure 1, that receptor-stimulated PtdIns breakdown might be an essential step in a mechanism of Ca^{2+} mobilization that is coupled to all Ca^{2+}-mobilizing receptors, in whatever organism or tissue they may occur (Michell 1975, 1979*a*, *b*; Michell *et al.* 1976*a*, *b*, 1977, 1979; Jones & Michell 1978*a*; Kirk *et al.* 1980; Michell & Kirk 1981*b*). Although this hypothesis has proved remarkably predictive and has therefore received considerable support (see, for example, Putney (1979, 1981), Berridge (1980), Gomperts *et al.* (1980) and Fain & Garcia-Sainz (1980)), it has been challenged on the basis

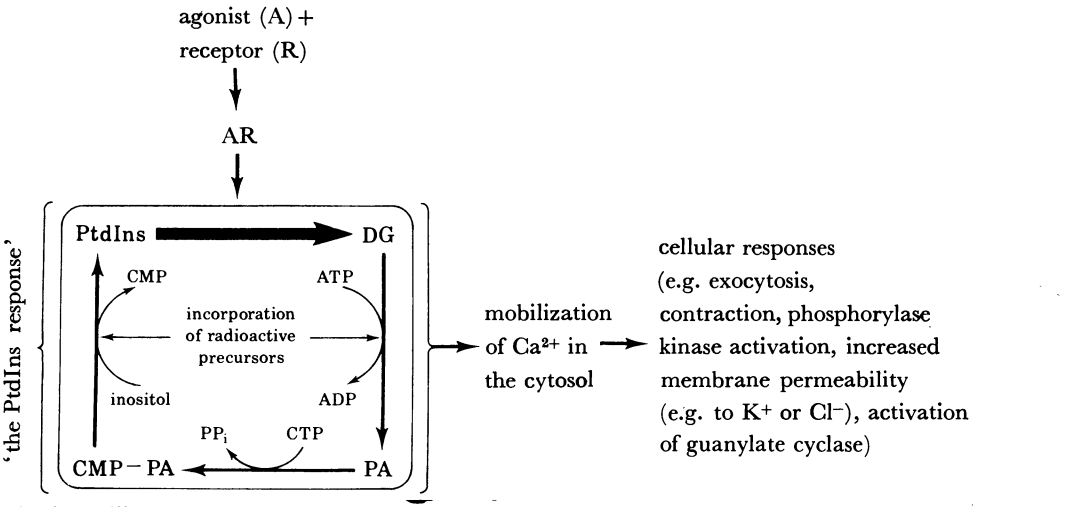

FIGURE 1. A scheme illustrating the function suggested for PtdIns breakdown as a coupling event in receptor-controlled mobilization of Ca^{2+} in the cytosol of many cells. The legend 'incorporation of radioactive precursors' within the cycle of the PtdIns response refers only to incorporation of precursors into the phosphorylinositol headgroup of the lipid: during synthesis *de novo* the diacylglycerol backbone is synthesized by pathways not shown here. Abbreviations: DG, 1,2-diacylglycerol; PA, phosphatidate; CMP-PA, phosphatidyl-CMP (also known as CDP-diacylglycerol or CMP-phosphatidate) (from Michell & Kirk 1981*b*). Compare figure 3 for another possible reaction sequence.

of recent information which suggests that PtdIns breakdown may sometimes be a response to, rather than independent of, receptor-stimulated Ca^{2+} mobilization (Cockcroft *et al.* 1980*a*, *b*; Cockcroft 1981).

The remainder of this paper will attempt to re-evaluate critically several of the most important characteristics of this response and to indicate important areas in which we lack vital information. When illustrative examples are presented, they will often be drawn from our recent studies of the isolated rat hepatocyte, a system that has proved extremely convenient for the study of the PtdIns response (Kirk *et al.* 1977, 1979, 1980, 1981*a*, *b*; Billah & Michell 1979; Michell *et al.* 1979; Tolbert *et al.* 1980).

Is inositol lipid breakdown evoked only and always by Ca^{2+}-mobilizing cell-surface receptors?

If inositol lipid breakdown is only involved in a mechanism for Ca^{2+} mobilization that can be switched on by any Ca^{2+}-mobilizing ligand, then a 'PtdIns response' should be evoked by all such receptors in all tissues in which they function. In addition, it should not be evoked by stimuli that employ other coupling mechanisms. The obvious analogy is with control of

adenylate cyclase, where all stimulatory and inhibitory receptors seem to act upon the same population of cyclase molecules, maybe through the same population of regulatory guanyl nucleotide binding/GTPase components.

At present, there are only two rapidly acting stimuli that might depart from the rule that inositol lipid breakdown is always a response to activation of a cell-surface receptor by a ligand that need not enter the target cell. One is the activation of phosphatidylinositol breakdown and labelling in islets of Langerhans by glucose and other insulin secretagogues. There is little doubt that the secretory response to glucose in this system is ultimately mediated by a rise in cytosol [Ca^{2+}], but it is not clear whether the stimulus for this Ca^{2+} mobilization is a cell-surface glucoreceptor or some intracellular metabolite of glucose (see, for example, Lazarus & Davis 1979). The second 'stimulus' that has no cell-surface receptor but which can evoke PtdIns breakdown in some, but not all, cells is the divalent cation ionophore A23187: discussion of this is deferred to a later section that deals with the role of Ca^{2+} in stimulation of PtdIns metabolism.

There are many cell-surface receptors for which the assertion that the PtdIns response is an accompaniment of Ca^{2+} mobilization seems unlikely to be contested: for example, α_1-adrenergic (in a variety of cells), H_1-histamine (smooth muscle), 5-HT_1 (blowfly salivary gland), V_1-vasopressin (liver, smooth muscle), ATP (liver) (Burgess *et al.* 1981; Kirk *et al.* 1981*a*), pancreozymin, pentagastrin and bombesin (pancreas), substance P (parotid, submaxillary and sublingual glands), thrombin and collagen (platelets), bradykinin (fibrosarcoma) (Bell *et al.* 1980), fMet-Leu-Phe (polymorphonuclear leucocytes), antigens and other secretagogues (mast cells). (For additional references, see the reviews by Michell (1975, 1979*a*, *b*), Jones & Michell (1978*a*), Michell & Kirk (1981*a*), Fain & Garcia-Sainz (1980) and Berridge (1980).)

But these past reviews also listed other receptors as evoking enhanced PtdIns turnover: what of these? One, the muscarinic cholinergic receptor, undoubtedly uses a rise in cytosol [Ca^{2+}] as its major intracellular messenger in many target cells (Hurwitz & Suria 1971; Triggle 1972; Michell 1979*a*, 1980). Whenever appropriate experiments have been done with these tissues there has always been an accompanying PtdIns response (see Michell (1980) and table II of Michell & Kirk (1981*a*)). However, there is now clear evidence that in some cells muscarinic receptors cause an inhibition of adenylate cyclase that is not Ca^{2+}-mediated (see, for example, Jacobs *et al.* (1980) and Berridge (1980)). Similarly, angiotensin both mobilizes Ca^{2+} (Keppens *et al.* 1977) and inhibits adenylate cyclase (Jard *et al.* 1981) in the liver. Unfortunately we still lack two pieces of information that are needed to reveal how the PtdIns response fits into this picture. It is not known whether, as with α_1- and α_2-adrenergic receptors, there are two functionally distinct types of muscarinic and angiotensin receptors that are not yet subject to discrimination by the available ligands. Nor do we know whether the muscarinic and angiotensin receptors that are negatively coupled to adenylate cyclase evoke a PtdIns response.

Some uncertainty also attends the cyclic nucleotide-independent PtdIns response that Lakshmanan (1978*a*, *b*; 1979) has demonstrated in pineal gland and sympathetic ganglia exposed to nerve growth factor (NGF) preparations. There are two reasons why this information should, for the moment, be treated with some reserve. First, NGF preparations are, unless isolated by particularly rigorous techniques, usually contaminated with renin activity (Avrith *et al.* 1980): the PtdIns response may therefore be a response to endogenously generated angiotensin. Secondly, there have been arguments presented both in favour of and against Ca^{2+} as a cellular mediator of the actions of NGF (Schubert *et al.* 1978; Landreth *et al.* 1980).

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Another ambiguous PtdIns response is that evoked by thyrotropin, since most of the effects of this hormone are undoubtedly mediated through activation of adenylate cyclase. However, it is clear that this PtdIns response is not dependent on cyclic AMP production (see Michell 1975). Intriguingly, there are other responses to thyrotropin that are independent of cyclic AMP and these appear to be Ca^{2+}-mediated (van Sande *et al.* 1975). Maybe it is to these that the PtdIns response is related, in which case one wonders whether there might be two distinct subclasses of thyrotropin receptors, one linked to adenylate cyclase and the other to Ca^{2+} mobilization?

Membrane depolarization produces PtdIns responses in ileum smooth muscle (Jafferji & Michell 1976*a*), ganglia (Nagata *et al.* 1973) and synaptosomes (Bleasdale & Hawthorne 1975; Pickard & Hawthorne 1978), but it is not yet certain whether these are primary responses to depolarization or secondary effects of endogenously released neurotransmitters. Of these three responses, interpretation of the PtdIns breakdown that occurs in electrically stimulated synaptosome beds seems the least unlikely to be confounded by such release (Pickard & Hawthorne 1978). In the ileum muscle it was shown that the observed response was not due to endogenous acetylcholine release (Jafferji & Michell 1976), but it now seems possible that some other excitatory neurotransmitter, maybe a peptide, could have been released from a nerve plexus to provoke the PtdIns turnover. Similarly, the persistence of a PtdIns response in denervated sympathetic ganglia (Nagata *et al.* 1973; see also Michell 1975) still leaves open the possibility of mediation by an excitatory transmitter derived from some internal neuronal circuit in the ganglion. This whole question therefore remains open.

Finally, there is the intriguing, and apparently unbroken, correlation between various types of proliferative stimuli and enhanced inositol lipid turnover (see Crumpton *et al.* 1975; Allan & Michell 1977; Diringer & Friis 1977; Michell 1979*b*; Hui & Harmony 1980; Hoffman *et al.* 1980). Again there is possibly a general correlation with functionally important changes in cellular Ca^{2+} status (Michell 1979*b*), but much more work is needed in this area.

What is the stimulated reaction that initiates the 'phosphatidylinositol response', and where in the cell does it happen?

Since 1974 there has been general agreement that stimulation of cells provokes PtdIns breakdown, and that other events such as phosphatidate and PtdIns labelling are secondary to this (see figure 1) (Hokin-Neaverson 1977; Michell *et al.* 1977; Jones *et al.* 1979). However, our studies of rat hepatocytes have recently raised the possibility that this ubiquitous disappearance of phosphatidylinositol might itself be secondary to the breakdown of phosphatidylinositol 4,5-bisphosphate (Kirk *et al.* 1981*a*).

Rat liver contains rather more than 2 μmol inositol per gram fresh tissue, of which about 80% is in the form of lipids extractable by acidified chloroform–methanol (Michell *et al.* 1970). Although almost all of this inositol lipid is phosphatidylinositol, there are small quantities of phosphatidylinositol 4-phosphate (PtdIns-4*P*) and phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5P_2): each constitutes maybe 1% of the total (Michell *et al.* 1970). Recently we have undertaken to check these figures by labelling liver to equilibrium with [^{3}H]inositol *in vivo*, isolating hepatocytes and estimating the distribution of [^{3}H]inositol among their lipids: again PtdIns-4*P* and PtdIns-4, 5P_2 each represented 1–2% of the total lipid inositol, with rather more of the former (L.M.J., unpublished data).

When hepatocytes are stimulated with sufficient vasopressin to saturate the V_1-receptors there

is rapid breakdown of all three inositol lipids: typical data for PtdIns and PtdIns-4,5P_2 are shown in figure 2. Similar, but smaller, effects are seen with α_1-adrenergic stimuli, angiotensin or ATP. Breakdown of PtdIns-4P and PtdIns-4,5P_2 can be detected within a few seconds and for each it amounts to 0.5–1 % of the total cell pool per second: extrapolation suggests that breakdown starts within 1 s of the addition of vasopressin (figure 2). PtdIns disappearance cannot be reliably established until about 5 min after stimulation, but extrapolation suggests that it also starts very soon after vasopressin is added and at a rate of about 1 % of the total cell pool per minute (figure 2). When the different concentrations of the three lipids are taken into account, it becomes apparent that the initial rates of breakdown of PtdIns and of the polyphosphoinositides (PtdIns-4P and PtdIns-4,5P_2) are quite similar (*ca.* 0.2–0.3 nmol g^{-1} tissue s^{-1}).

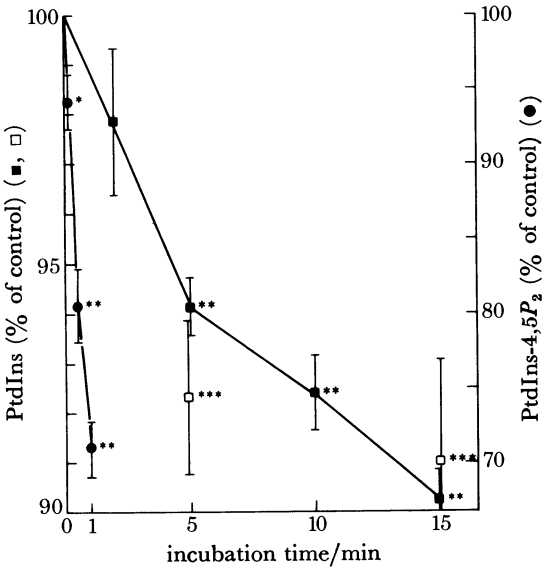

FIGURE 2. Time-courses of the breakdown of PtdIns and of PtdIns-4,5P_2 in rat hepatocytes exposed to 0.23 μM [Arg^8]-vasopressin. Breakdown of PtdIns was assayed as the decrease in ^{3}H-labelled lipids in hepatocytes labelled to equilibrium with [^{3}H]inositol *in vivo* (■) or as the decrease in PtdIns concentration in stimulated cells (□): both are from Kirk *et al.* (1981*b*). PtdIns-4,5P_2 breakdown (●) was assayed as the loss of ^{32}P from PtdIns-4,5P_2 in cells previously labelled by incubation with $^{32}P_i$ for 1 h. (Data from Kirk *et al.* (1981*a*.) All values are mean ± s.e.m. Significant differences from control are indicated: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$.

If PtdIns breakdown were a primary event in receptor coupling then it should occur at the plasma membrane, but attempts to determine the cellular site of the loss of PtdIns in stimulated cells have given variable results. The loss appeared to be from secretory vesicle membranes in electrically stimulated synaptosomes (Pickard & Hawthorne 1978) and in glucose-stimulated islets of Langerhans (Clements *et al.* 1977), from endoplasmic reticulum in acetylcholine-stimulated pancreas and from the plasma membrane in acetylcholine-stimulated salt gland (Hokin-Neaverson 1977). By contrast, attempts to find a defined location for the loss of PtdIns in either vasopressin-stimulated hepatocytes (Kirk *et al.* 1981*b*) or acetylcholine-stimulated adrenal medulla (Azila & Hawthorne 1981) have failed. It seems likely that the PtdIns response has a similar mechanism in each tissue, so this variety of results is puzzling. One explanation might be that the initial response to stimulation is PtdIns-4,5P_2 breakdown, so that the regularly observed PtdIns 'breakdown' is itself a secondary event that simply represents the continuous

intracellular movement and consumption of cellular PtdIns for the synthesis of PtdIns-4,5P_2 via PtdIns-4P (figure 3). We are now attempting to make direct determinations of the subcellular distributions of PtdIns-4,5P_2 and PtdIns-4P in stimulated and unstimulated hepatocytes. Existing information on the subcellular location of the polyphosphoinositides (Michell 1975; Griffin & Hawthorne 1978) suggests that they might be located and degraded at the plasma membrane, where they could readily play a role in receptor–response coupling.

The route of stimulated PtdIns-4,5P_2 breakdown is still unknown. However, past experience of a variety of systems points to breakdown of an inositol lipid to diacylglycerol, thence returning via phosphatidate and phosphatidyl-CMP to PtdIns (see Michell (1975) and Michell *et al.* (1977)). If PtdIns-4,5P_2 were to break down by stimulation of its dephosphorylation to PtdIns-4P and PtdIns, the obvious alternative to this phosphodiesterase route, then the

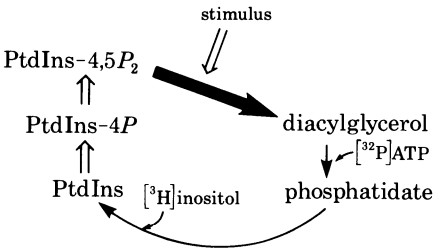

FIGURE 3. A possible alternative scheme for the reaction sequence involved in the PtdIns response. In the usual view (figure 1), PtdIns breakdown is seen as the initiating event. In this alternative suggestion, receptors would primarily exert control over a PtdIns-4,5P_2 phosphodiesterase, probably at the plasma membrane, and the disappearance of PtdIns would be a consequence of its consumption during the resynthesis of PtdIns-4,5P_2.

concentration of PtdIns-4P should rise rather than fall, the effect upon PtdIns concentrations would be undetectable, and diacylglycerol and phosphatidate would not be produced: none of these predictions are fulfilled. Hence we suspect that stimulated breakdown of PtdIns-4,5P_2 is catalysed by a phosphodiesterase, with the release of inositol-1,4,5P_3. This extremely polar molecule is rapidly degraded by removal of its 5-phosphate or of its 4- and 5-phosphates, at least in erythrocytes (Downes & Michell 1981; C.P.D., M. C. Mussat & R.H.M., unpublished data) and iris muscle (Akhtar & Abdel-Latif 1980).

How close is the coupling between receptor occupation and the breakdown of inositol lipids?

The initial reports that pointed out the similarity between the dose–response curves describing ligand binding to receptors and stimulation of PtdIns metabolism drew mainly upon studies of muscarinic cholinergic stimulation of PtdIns labelling (Michell *et al.* 1976*a*, *b*). Even then, however, the only available information on stimulated PtdIns breakdown seemed to conform to the same general pattern (Hokin-Neaverson 1974; Jones & Michell 1974; Michell *et al.* 1976*a*). Little further information on this aspect of cholinergic responses has since emerged, except for a recent report by Weiss & Putney (1981) that describes a parotid PtdIns labelling response at untypically low concentrations of agonist (cf. table 8.3 of Michell *et al.* 1976*b*); interpretation of this result must, however, be suspended until equivalent measurements are made of inositol lipid breakdown.

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An additional system at which there appears to be an invariant association between PtdIns breakdown and Ca^{2+} mobilization, but with the dose–response curve for PtdIns breakdown markedly displaced to higher agonist concentrations than are needed for the electrophysiological response, is the 5-HT_1 receptor of blowfly salivary glands (Fain & Berridge 1979; Berridge 1980, 1981)). This again has been interpreted as evidence for a close relationship between PtdIns breakdown and Ca^{2+} mobilization, with the more sensitive electrophysiological response a consequence of changes in cytosol [Ca^{2+}].

A further test of the idea that inositol lipid breakdown is closely coupled to receptor occupation was recently possible in rat hepatocytes. Here the dose–response curve for activation

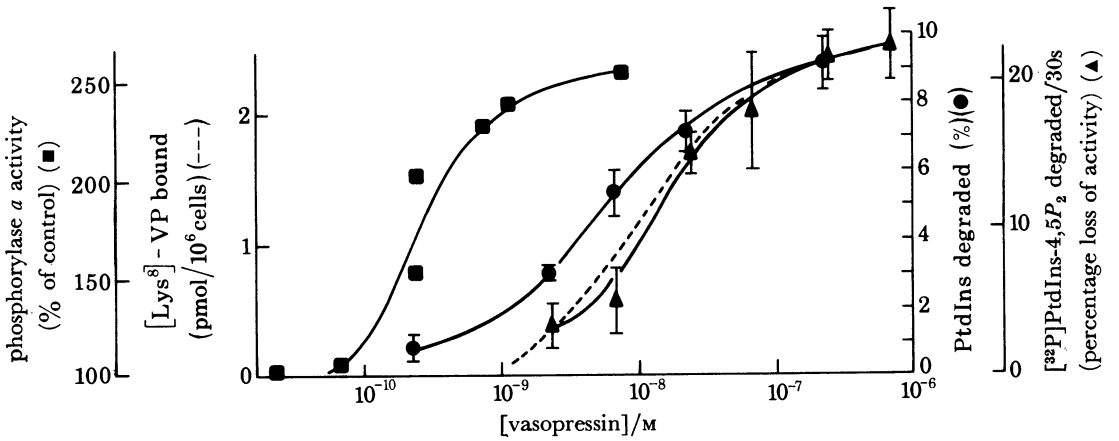

FIGURE 4. The relation between receptor occupation and responses at the V_1-vasopressin receptor of rat hepatocytes. The broken line depicts occupation of receptors by [Lys^8]-vasopressin (Cantau *et al.* 1980). PtdIns breakdown (●) was assayed by loss of [^{3}H]inositol from the lipids of hepatocytes labelled with [^{3}H]inositol *in vivo* and stimulated with [Arg^8]-vasopressin for 15 min (Kirk *et al.* 1981*b*). PtdIns-4,5P_2 breakdown (▲) was assayed during stimulation for 1 min of hepatocytes previously labelled for 60 min with $^{32}P_i$ (Kirk *et al.* 1981*a*). Data on activation of phosphorylase (■) are from Kirk *et al.* (1979).

of glycogen phosphorylase by [Arg^8]-vasopressin, a response mediated by cytosol Ca^{2+}, is displaced to a 40-fold lower concentration than the curve that describes binding of [Lys^8]-vasopressin to the V_1-vasopressin receptor (Kirk *et al.* 1979; Cantau *et al.* 1980); [Arg^8]- and [Lys^8]-vasopressin are equipotent at this receptor. In 1979, Kirk *et al.* suggested that there was a 'receptor reserve' for the Ca^{2+}-mediated physiological response but not for the putatively receptor-coupled PtdIns labelling response. Recently this conclusion has been given much greater strength by the determination of dose–response curves for V_1-receptor-activated PtdIns breakdown and PtdIns-4,5P_2 breakdown (Kirk *et al.* 1981*a*, *b*). It appears that both of these curves are essentially identical to the receptor occupation curve (figure 4).

It should be noted, however, that there is no principle that would suggest that these differences in response between stimulated inositol lipid breakdown and Ca^{2+}-mediated physiological responses should constitute a universal pattern. Sometimes a full physiological response will require activation of the entire receptor population, in which case the dose–response curves for receptor occupation, inositol lipid breakdown and physiological responses should be superimposed: the substance P receptor of rat parotid gland may be of this type (Hanley *et al.* 1980; Weiss & Putney 1981).

If it is assumed that the details of receptor–response coupling at any individual type of receptor are invariant, then an interesting corollary of this argument emerges: in different tissues that have different numbers of a particular receptor the maximum rate of the initiating inositol lipid breakdown step should vary in proportion to the number of functional receptors, but without any change in the position of the dose–response curve. Physiological responses, by contrast, should show a large receptor reserve in tissues with many receptors and none in those with few receptors. No rigorous test of this notion will be possible until we know the initiating reaction of the PtdIns response, but existing information on V_1-vasopressin receptors in rat hepatocytes and aorta appears compatible with the expected pattern. Receptor binding and all measures of the PtdIns response in these tissues show half-maximal levels in a very narrow concentration range between 2.5 and 6 nM [Arg^8]-vasopressin (Kirk *et al.* 1979, 1981*a*, *b*; Takhar & Kirk 1981; Cantau *et al.* 1980): these figures do not yet include information on inositol lipid breakdown in aorta. By contrast, the dose–response curves for hepatic glycogen phosphorylase activation and for aortic contraction are very different, with the former showing a 40-fold receptor reserve (figure 4) and the latter apparently none (Altura 1974; Takhar & Kirk 1981).

Possible interpretations of the apparent close coupling between receptor occupation and the phosphatidylinositol response

In principle, there are three possible explanations for the universal association between activation of Ca^{2+}-mobilizing receptors and the stimulation of inositol lipid breakdown, regardless of whether the primary breakdown is of PtdIns or PtdIns-4,5P_2.

In the first, inositol lipid breakdown would be coupled closely to activation of all receptors that control Ca^{2+} mobilization but be quite unrelated to cellular Ca^{2+} homoeostasis (i.e. alternative *a* in Figure 5 of Michell 1975). As a hypothesis, this notion is valueless since it begets no firm predictions. It also seems improbable in evolutionary terms (Michell 1975) and will therefore not be considered further at present.

The second possible explanation is that the PtdIns response is initiated by the rise in cytosol [Ca^{2+}] caused by receptor activation. If that were so, then our knowledge of the closeness of coupling between receptor occupation and the PtdIns response, as contrasted with the variable relation between receptor occupation and Ca^{2+}-mediated physiological responses, should allow firm predictions about its behaviour (Jafferji & Michell 1976; Michell & Kirk 1981). Before making such predictions we must state an assumption, namely that if inositol lipid breakdown and 'physiological responses' (e.g. activation of glycogen phosphorylase) were both simultaneously controlled by receptor-stimulated changes in cytosol [Ca^{2+}] then both would experience identical temporal changes in cytosol [Ca^{2+}].

The simple case is that in which the dose–response curves of agonist binding, the PtdIns response and physiological responses are the same (e.g. the response of the parotid gland to substance P?). In such situations, the sensitivities to Ca^{2+} of the physiological response and of inositol lipid breakdown should be identical, so any protocol designed to modify cytosol [Ca^{2+}] should modify both in identical ways. More complex cases are those in which physiological responses show a receptor reserve but inositol lipid breakdown does not: examples would include the vasopressin receptor of liver (figure 3) and the muscarinic cholinergic receptors of exocrine pancreas (M. R. Hokin 1968; Hokin-Neaverson 1974, 1977) and of ileum smooth

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muscle (Jafferji & Michell 1976*b*). For these it would seem likely that existence of an *n*-fold receptor reserve for a particular physiological response would mean that activation of inositol lipid breakdown requires *n*-fold more cytosol Ca^{2+} than does activation of that physiological response. This being so, an inevitable deduction is that procedures designed to suppress changes in cytosol [Ca^{2+}] should suppress inositol lipid breakdown more readily than they would suppress 'physiological' Ca^{2+}-mediated responses (Jafferji & Michell 1976*b*; Michell & Kirk 1981*b*). In addition, it would be correspondingly more difficult, when using agents such as ionophores, to elevate cytosol [Ca^{2+}] to levels adequate to elicit inositol lipid breakdown than to levels capable of evoking physiological events (Michell & Kirk 1981*b*).

The third alternative, which has been our working hypothesis for several years, is that inositol lipid breakdown is indeed an essential step in the process by which receptors bring about the mobilization of Ca^{2+} in the cytosol of stimulated cells. Many predictions spring from this hypothesis, some of them already tested to some degree (e.g. that there should be an unbroken correlation between the occurrence of receptor-stimulated inositol lipid breakdown and receptor-stimulated Ca^{2+} mobilization) and others not yet amenable to direct test (e.g. that non-specific activation and inhibition of the receptor-coupled mechanism that catalyses inositol lipid breakdown should, respectively, evoke and antagonize 'normal' physiological responses to receptor activation). One of the key experiments that could, in principle, disprove this hypothesis would be a demonstration that all of the changes in inositol lipid metabolism in a particular stimulated cell were consequences of the receptor-stimulated Ca^{2+} mobilization, i.e. that all inositol lipid breakdown was 'just another Ca^{2+}-mediated physiological response', but one of unknown function.

As will be clear from the preceding paragraphs, some of the most important and easily accessible experimental tests of the last two alternatives are encompassed in studies designed to probe the role or lack of role of Ca^{2+} ions in the mediation of receptor-stimulated inositol lipid breakdown. The last section will deal with these studies, since they undoubtedly constitute the focus for the most important disagreements in this field at present.

What role does Ca^{2+} play in receptor-stimulated phosphatidylinositol metabolism?

In principle, *each individual enzymatic step* involved in changes in inositol lipid metabolism is classifiable into one of four groups on the basis of its responsiveness to changes in cytosol [Ca^{2+}]:

(1) events that are not influenced by changes in cytosol [Ca^{2+}];

(2) events that are controlled by fluctuations in cytosol [Ca^{2+}];

(3) events that are wholly controlled by some influence other than cytosol [Ca^{2+}] (e.g. an activated receptor), but which display an essential requirement for Ca^{2+} that is satisfied even at the cytosol [Ca^{2+}] that prevails in unstimulated cells;

(4) events whose control is a composite of the effects of [Ca^{2+}] and of some other factor(s), (i.e. of categories 2 and 3 above).

Most previous discussions (e.g. Michell 1975; Michell *et al.* 1977; Berridge 1980; Cockcroft 1981) have considered only the first two of these alternatives (see Michell & Kirk 1981*b*). For example, Cockcroft (1981) explicitly assigns all of the PtdIns responses of a variety of cells to category 1, category 2 or a combination of both. Moreover, these discussions have not distinguished clearly between inferences drawn from studies of inositol lipid breakdown and

of labelling, despite the fact that whenever an assay of the PtdIns response depends on several enzymatic steps then each step is individually classifiable into one of these four categories. For this reason, attempts to use studies of the labelling of PtdIns (a minimum of four reactions) to determine the role of Ca^{2+} are intrinsically ambiguous and should be abandoned (Michell & Kirk 1981*a*). Although data derived from such studies provided some of the most useful early clues (see, for example, Trifaró (1969*b*) and Oron *et al.* (1975)), they will not be considered in this discussion. Another oversight in most past discussions has been failure to acknowledge that stimulation of a single cell might simultaneously evoke more than one effect upon inositol lipid metabolism: acknowledgement of this potential complexity, for example by Abdel-Latif *et al.* (1978), Egawa *et al.* (1981) and Lapetina *et al.* (1981), represents an important step forward.

Table 1 summarizes those studies that have attempted some type of experimental assessment of the role of cytosol [Ca^{2+}] in controlling the breakdown either of PtdIns or of polyphosphoinositides in stimulated cells.

Considering PtdIns first, it becomes clear that many permutations of tissue, agonist, A23187 and low-Ca^{2+} media have given varied results. In several tissues (parotid and lacrimal glands, hepatocytes, insect salivary gland, synaptosomes) the available evidence points clearly to the view that changes in cytosol [Ca^{2+}] are irrelevant for the demonstration of stimulated PtdIns breakdown. In at least three of these (hepatocytes, blowfly salivary gland, ACh-stimulated parotid gland) physiological responses exhibit a substantial receptor reserve. Despite this, PtdIns is in all cases much more resilient to Ca^{2+} deprivation (see above). In platelets (see Rittenhouse-Simmons & Deykin 1981; Broekman *et al.* 1980) and in vas deferens the initial rates of receptor-stimulated PtdIns breakdown are unchanged by Ca^{2+} deprivation. Again this occurs despite the fact that the receptor reserve of vas deferens would predict that any receptor-stimulated PtdIns breakdown that was Ca^{2+}-mediated should be very sensitive to Ca^{2+} deprivation. Thus it seems unlikely that either the platelet or vas deferens response is Ca^{2+}-mediated. However, in both of these cells there is also unambiguous evidence for Ca^{2+}-stimulated PtdIns breakdown, thus raising the possibility that two separate routes for PtdIns breakdown coexist. Indeed, this is the conclusion drawn by Egawa *et al.* (1981) for vas deferens. Whether it also applies to platelets is disputed: Lapetina *et al.* (1981) found that diacylglycerol released from PtdIns by thrombin is more readily converted to phosphatidate than is that liberated by A23187, but Bell & Majerus (1980) found no evidence for two functionally distinguishable pools. Phytohaemagglutinin-stimulated PtdIns breakdown in lymphocytes continues for several hours even in cells incubated in EDTA, albeit at a reduced rate, so mediation of the receptor-controlled event by Ca^{2+} again seems unlikely (Hui & Harmony 1980). Exocrine pancreas offers data sufficiently contradictory for no conclusion to be possible at present. Farese *et al.* (1980) claim that both acetylcholine-stimulated PtdIns breakdown and amylase secretion are abolished by Ca^{2+}-deprivation and mimicked by A23187. However, Hokin-Neaverson (1977) states that A23187 does not provoke PtdIns breakdown.

Finally we must consider fMet-Leu-Phe-stimulated PtdIns breakdown in the polymorphonuclear leucocyte, the only cell for which current evidence makes a reasonable case in favour of the entire PtdIns response being Ca^{2+}-mediated (contrast Cockcroft (1981) who ascribes five different cell types to this category). In this cell, pre-labelled PtdIns is rapidly broken down in response to either fMet-Leu-Phe or a Ca^{2+} ionophore (Cockcroft *et al.* 1980). In addition,

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TABLE 1. THE ROLE OF Ca^{2+} IN RECEPTOR-STIMULATED BREAKDOWN OF INOSITOL LIPIDS

tissue	receptor-directed stimulus	lipid broken down	effect of Ca^{2+} deprivation	evoked by A23187 and Ca^{2+}?	references
parotid gland	acetylcholine	PtdIns	unchanged	no	Jones & Michell (1975)
	adrenalin	PtdIns	unchanged	no	Jones & Michell (1975)
	substance P	PtdIns	unchanged	no	Jones & Michell (1978*b*)
blowfly salivary gland	5-hydroxytrypt-amine	PtdIns	unchanged	no	Fain & Berridge (1979), Berridge (1980, 1981)
hepatocytes	vasopressin	PtdIns	unchanged	no	Billah & Michell (1979), C. J. K. (unpublished)
lacrimal gland	acetyl-β-methyl-choline	PtdIns	unchanged	?	Jones *et al.* (1979)
synaptosomes	electrical polar-ization	PtdIns	unchanged	no	M. R. Pickard & J. N. Hawthorne (personal communication), Griffin & Hawthorne (1978)
platelets	thrombin	PtdIns	unchanged	yes	Rittenhouse-Simmons (1981), Rittenhouse-Simmons & Deykin (1981), Lapetina *et al.* (1981)
vas deferens	acetylcholine	PtdIns	unchanged	yes	Egawa *et al.* (1981)
lymphocytes	phytohaemagglutinin	PtdIns	decreased	?	Hui & Harmony (1980)
pancreas	acetylcholine	PtdIns	abolished	yes or no	Hokin-Neaverson (1977), Farese *et al.* (1980)
polymorphonuclear leucocytes	fMet-Leu-Phe	PtdIns	abolished	yes	Cockcroft *et al.* (1980)
hepatocyte	vasopressin	PtdIns-4,5P_2 (and PtdIns-4P)	decreased	no	Kirk *et al.* (1981*a*)
parotid gland	carbamylcholine	PtdIns-4,5P_2 (and PtdIns-4P)	unchanged	no	L. M. J. (unpublished), J. W. Putney & S. J. Weiss (personal communication)
iris muscle	acetylcholine or adrenalin	PtdIns-4,5P_2	abolished	yes	Abdel-Latif *et al.* (1978)
erythrocyte	none known	PtdIns-4,5P_2 (and PtdIns-4P)	—	yes	Lang *et al.* (1977)
synaptosomes	none known	PtdIns-4,5P_2 (and PtdIns-4P)	—	yes	Griffin & Hawthorne (1978)

this breakdown, measured at 10 s, appears to be abolished after incubation in a Ca^{2+}-free medium, despite the fact that about one-fifth of the normal secretory response is still expressed under these conditions. From this result, Cockcroft *et al.* (1980) concluded that 'breakdown of PtdIns is not essential for Ca^{2+} mobilization and secretion'. Cockcroft (1981) then rightly claimed that any one such demonstration of a receptor-triggered and Ca^{2+}-mediated physiological response that was unaccompanied by any PtdIns breakdown would negate the general hypothesis that PtdIns breakdown is essential to some universal receptor-controlled Ca^{2+}-mobilizing mechanism: she illustrated this by reference to Popper's example of the single black swan that disproves the general hypothesis that all swans are white. However, she then wrongly stated that 'any example of Ca^{2+}-dependent PtdIns turnover would resemble the "black swan"'. It would not. In this context the only unambiguous 'black swan' is a stimulated cell in which no component of receptor-stimulated PtdIns breakdown (or maybe of PtdIns-4,5P_2 breakdown) is controlled in any way other than via Ca^{2+}: only this rigorous definition can avoid the doubt introduced by 'very dirty swans' (for example, flawed conclusions arising from cells where both receptors and Ca^{2+} can cause PtdIns breakdown, or from measurements of PtdIns labelling). At the moment the published information on the polymorphonuclear leucocyte is not complete enough for the true colour of its feathers to be certain, and additional data will be awaited with interest.

Earlier we raised the possibility that the primary receptor-controlled event might be PtdIns-4,5P_2 breakdown rather than PtdIns breakdown, but available information on the cellular control of polyphosphoinositide breakdown is even more confused than for PtdIns (table 1). The very rapid vasopressin-stimulated breakdown in the hepatocyte appears not to be Ca^{2+}-mediated: despite its requirement for occupation of far more receptors than activation of glycogen phosphorylase, it is still much more resilient to Ca^{2+} deprivation (see above). In A23187-treated synaptosomes, polyphosphoinositide breakdown is rapid and dependent on Ca^{2+} (Griffin & Hawthorne 1978). However, the only 'physiological' stimulus that has been tested on synaptosomes (high extracellular $[K^+]$) evokes no breakdown despite raising cytosol $[Ca^{2+}]$ sufficiently to evoke secretion (Griffin *et al.* 1980). A second system in which polyphosphoinositide breakdown can be switched on by Ca^{2+} is the erythrocyte, where the enzyme responsible is a membrane-bound polyphosphoinositide phosphodiesterase (Allan & Michell 1978; Downes & Michell 1981*a*). Initially both our studies and those of D. Allan & M. Thomas (personal communication) suggested that this phosphodiesterase had a sensitivity to Ca^{2+} such that it might be activated in hormone-stimulated cells, but recent studies have shown this to be incorrect: when assayed at cytoplasmic ionic strength and $[Mg^{2+}]$ it showed no activity at $[Ca^{2+}]$ below 100 μM (Downes & Michell 1981*b*). Finally, the PtdIns-4,5P_2 breakdown provoked by intense muscarinic cholinergic or α_1-adrenergic stimulation of iris muscle does appear to be Ca^{2+}-mediated, but is delayed long beyond evocation of the contractile response (Abdel-Latif *et al.* 1978).

Two clear verdicts can come from these limited studies of the stimulated breakdown of polyphosphoinositides: (1) we know too little to draw firm conclusions about its normal control; and (2) future studies of stimulated inositol lipid breakdown will ignore this process at their peril. However, careful experimental design may be needed to detect these very rapid effects: for example, an earlier study of hepatocytes (Tolbert *et al.* 1980) failed to detect this response to Ca^{2+}-mobilizing agonists.

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Conclusion

Stimulated inositol lipid metabolism is a universal cellular correlate of the activation of receptors whose central effect is to elevate the cytosol [Ca^{2+}] in target cells. Initiation of this response appears to involve activation of a phosphodiesterase but it is not yet clear whether this enzyme attacks PtdIns or PtdIns-4,5P_2. In some cells inositol lipid breakdown appears to be activated by receptors without the intervention of Ca^{2+}, in others a major component of inositol lipid breakdown may be a consequence of the mobilization of cytosolic Ca^{2+} by receptors, and in yet others both of these mechanisms may make large contributions. The realization that inositol lipid breakdown may be provoked by at least two distinct mechanisms introduces new difficulties into the design of definitive experimental tests of the hypothesis that receptors may employ inositol lipid breakdown as a reaction essential to coupling between receptor activation and Ca^{2+} mobilization. None of the evidence yet available is adequate to falsify this general hypothesis. To facilitate future progress, analyses of the mechanism and function of stimulated inositol lipid metabolism should focus upon the breakdown of inositol lipids, rather than their labelling.

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