Phosphatidylinositol Metabolism in Rat Hepatocytes Stimulated by Glycogenolytic Hormones

EFFECTS OF ANGIOTENSIN, VASOPRESSIN, ADRENALINE, IONOPHORE A23187 AND CALCIUM-ION DEPRIVATION

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1. The effects on phosphatidylinositol metabolism of three Ca^{2+} -mobilizing glycogenolytic hormones, namely angiotensin, vasopressin and adrenaline, have been investigated by using rat hepatocytes. 2. All three hormones stimulate both phosphatidylinositol breakdown and the labelling of this lipid with ^{32}P . 3. The response to angiotensin occurs quickly, requires a high concentration of the hormone and is prevented by [1-sarcosine, 8-isoleucine]angiotensin, a specific angiotensin antagonist that does not prevent the responses to vasopressin and to adrenaline. This response therefore seems to be mediated by angiotensin-specific receptors. 4. [1-Deaminocysteine, 2-phenylalanine, 7-(3,4-dihydroxyproline), 8-arginine]vasopressin, a vasopressin analogue with enhanced anti-diuretic potency, is relatively ineffective at stimulating phosphatidylinositol metabolism. This suggests that the hepatic vasopressin receptors that stimulate phosphatidylinositol breakdown are different in their ligand selectivity from the antidiuretic vasopressin receptors that activate renal adenylate cyclase. 5. Incubation of hepatocytes with ionophore A23187, a bivalent-cation ionophore, neither mimicked nor appreciably changed the effects of vasopressin on phosphatidylinositol metabolism, suggesting that phosphatidylinositol breakdown is not controlled by changes in the cytosol Ca^{2+} concentration. This conclusion was supported by the observation that hormonal stimulation of phosphatidylinositol breakdown and resynthesis persists in cells incubated for a substantial period in EGTA, although this treatment somewhat decreased the phosphatidylinositol response of the hepatocyte. The phosphatidylinositol response of the hepatocyte therefore appears not to be controlled by changes in cytosol $[\text{Ca}^{2+}]$, despite the fact that this ion is thought to be the second messenger by which the same hormones control glycogenolysis. 6. These results may be an indication that phosphatidylinositol breakdown is an integral reaction in the stimulus-response coupling sequence(s) that link(s) activation of α -adrenergic, vasopressin and angiotensin receptors to mobilization of Ca^{2+} in the rat hepatocyte.

A specific increase in the metabolism of phosphatidylinositol is a widespread cellular response to activation of certain types of cell-surface receptor for hormones and neurotransmitters, particularly receptors in whose actions Ca^{2+} ions are strongly implicated. One possible explanation of this association could be that phosphatidylinositol breakdown, the reaction controlled by receptor activation, is somehow implicated in the mechanism(s) through which various types of receptor control the mobilization of Ca^{2+} ions within cells (Michell, 1975, 1979; Jones & Michell, 1978a; Jones *et al.*, 1979). Most studies of this phenomenon have so far been carried out in various types of tissue fragments, particularly

from secretory tissues and smooth muscles. More recently, however, it has become clear that rat hepatocytes possess at least three populations of hormone receptors in whose actions Ca^{2+} ions are intimately involved, namely those for α -adrenergic ligands, vasopressin and angiotensin (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Hems *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1977; Van de Werve *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978). It has been known for some years that α -adrenergic stimulation brings about enhanced phosphatidylinositol metabolism in a variety of tissues, possibly including rat liver (De Torrentegui & Berthet, 1966; Jones & Michell, 1978a), and consideration of the effects of vasopressin and angiotensin on smooth muscles suggested to us that their actions might also involve phosphatidylinositol

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metabolism (Michell *et al.*, 1977a). Subsequently it was found that isolated rat hepatocytes show a rapid increase in the labelling of their phosphatidylinositol with ^{32}P when exposed to vasopressin (Kirk *et al.*, 1977, 1978, 1979; Billah & Michell, 1978) or angiotensin (Billah & Michell, 1978). The present paper is concerned with detailed description of the response to angiotensin, with demonstration of the fact that the phosphatidylinositol responses of hepatocytes involve phosphatidylinositol breakdown and with the relationship between the simultaneous changes in cellular Ca^{2+} status and in phosphatidylinositol metabolism that these hormones provoke.

Materials and Methods

Materials

Bovine plasma albumin (fraction V; fatty acid-poor, dialysed against 0.154M-NaCl for at least 24h) was from Miles Laboratories., Stoke Court, Stoke Poges, Slough SL2 4LY, Berks., U.K.

Adrenaline, collagenase (type 1) and synthetic vasopressin (grade V) were from Sigma Chemical Co., Poole, Dorset BH17 7NH, U.K.

Synthetic angiotensin octapeptide and [1-sarcosine-8-isoleucine]angiotensin were from Peninsula Laboratories Inc., P.O. Box 1111, San Carlos, CA 94070, U.S.A.

Bivalent-cation ionophore A23187 was a gift from Eli-Lilly, Indianapolis, IN, U.S.A.

Sagatal (pentobarbitone sodium; 60mg/ml) was from May and Baker, Dagenham, Essex, U.K.

[1-Deaminocysteine,2-phenylalanine,7-(3,4-didehydroproline),8-arginine]vasopressin was a gift from Dr. Roderich Walter, Department of Biochemistry, University of Chicago, Chicago, IL, U.S.A.

Isolation of rat hepatocytes

Hepatocytes were isolated from fed male Wistar rats (230–260g) essentially by the method of Berry & Friend (1969). A rat was anaesthetized with 0.4ml of Sagatal and the liver was cannulated via the portal vein. After washing out the blood with Ca^{2+} -free Krebs–Ringer bicarbonate buffer, pH7.4, the liver was subjected to recirculating perfusion with 100ml of the same buffer containing 30mg of collagenase. The perfusion was at 37°C and the medium was constantly gassed with O_2/CO_2 (19:1, v/v). After 20–30min, the collagenase-treated liver was gently stirred in a plastic beaker with a plastic rod. The separated cells were washed 3–4 times by suspension in Krebs–Ringer–Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; 25mM] buffer, pH7.4, containing 1mM- Ca^{2+} , so as to remove debris and broken cells. When viewed by phase-contrast microscopy, approx. 90% of the cells remained non-

aggregated and phase-dense and also excluded Trypan Blue. Cells isolated from 24h-starved rats by this method synthesized glucose from 10mM-pyruvate or 10mM-alanine at rates comparable with those reported by other workers (e.g. Berry & Friend, 1969).

Incorporation of ^{32}P into hepatocyte lipids *in vitro*

Isolated washed cells were diluted to a dry weight of approx. 20mg/ml with Krebs–Ringer–Hepes buffer containing 1mM- Ca^{2+} and 4% (w/v) bovine serum albumin (unless otherwise stated). A portion (0.5ml) of this suspension was then added by using an automatic pipette to plastic vials each containing 0.5ml of the same buffer containing 40 μCi of [^{32}P]P_i and appropriate other additions (e.g. hormones). The incubations were usually for 15min and incorporation was stopped by adding 3.75ml of chloroform/methanol (1:2, v/v). Lipids were extracted and separated by paper chromatography and the separated phosphatidylinositol and, in some experiments, the combined spot containing most other phospholipids were analysed for radioactivity (see Jones *et al.*, 1979). The labelling of the lipids in stimulated cells was expressed as a percentage of the value in incubations without added hormones. In each experiment, two to four replicate assays were carried out under each set of incubation conditions.

Studies of phosphatidylinositol breakdown

For some of the experiments on phosphatidylinositol breakdown, prelabelled hepatocytes were isolated from rats that had received an intraperitoneal injection of approx. 0.7mCi of [^{32}P]P_i about 24h previously. Incubation conditions were identical with those in experiments for ^{32}P incorporation. Both in control and stimulated cells, the separated phosphatidylinositol was then analysed for radioactivity. In some experiments, both with radioactive and non-radioactive cells, the phosphate content of the lipids was estimated. The results were expressed as percentage changes in the ^{32}P or phosphate contents of the lipids relative to controls run in parallel. In each experiment, six to 18 replicate assays were done under each set of incubation conditions.

Results

Stimulation of phosphatidylinositol labelling by angiotensin

Incubation of hepatocytes with [^{32}P]P_i in the presence of angiotensin produced a dose-dependent increase in the labelling of phosphatidylinositol

(Fig. 1), but little change in the labelling of the other phospholipids (results not shown). Half-maximum stimulation occurred at about 50 nM-angiotensin, a much higher concentration than is needed for half-maximum stimulation of glycogen phosphorylase (Keppens & De Wulf, 1976). When hepatocytes were incubated with ^{32}P for 5 min and angiotensin was then added, an increase in phosphatidylinositol labelling was seen within 1 min and the maximum stimulated rate of incorporation was achieved in less than 5 min (Fig. 2).

When individual preparations of hepatocytes were incubated with 1 μM -angiotensin, vasopressin (10 m-units/ml; 23 nM) and 100 μM -adrenaline, concentrations of ligands that give close to maximum stimulation of phosphatidylinositol labelling, we routinely observed that the stimulation in phosphatidylinositol labelling during 15 min incubations was higher with vasopressin (5–8-fold) than with angiotensin or adrenaline (both 3–5-fold) (e.g. Table 2).

De Torrentegui & Berthet (1966) and Kirk *et al.* (1977) have shown that the adrenaline response is susceptible to blockade by α -adrenergic antagonists.

Fig. 1. Dose-response curve of angiotensin-stimulated phosphatidylinositol labelling

Hepatocytes were incubated with ^{32}P for 15 min in the presence of angiotensin. Results are the means for triplicate incubations in a single experiment, one of three that gave similar results.

Kirk *et al.* (1977) also showed that an α -antagonist did not inhibit the vasopressin response, thus implicating a distinct population of vasopressin receptors in this response. To determine whether the response to angiotensin is controlled through a third discrete receptor population we have employed the angiotensin-receptor antagonist [1-sarcosine, 8-isoleucine]angiotensin. This inhibited the phosphatidylinositol response to angiotensin in a dose-dependent manner (Fig. 3), but it did not inhibit the responses evoked by adrenaline and vasopressin (results not shown). It therefore appears that the angiotensin-induced enhancement in the incorporation of ^{32}P into phosphatidylinositol is mediated through angiotensin-specific receptors and that these are distinct from the α -adrenergic and vasopressin receptors whose activation can produce the same type of response.

Stimulation of phosphatidylinositol breakdown

There are now a considerable number of situations in which it has been clearly shown that receptor-stimulated phosphatidylinositol metabolism involves phosphatidylinositol breakdown, and it seems likely that this is the initial reaction in all such responses (see Jones *et al.*, 1979). This also appears to be the case in the responses of hepatocytes. Table 1 summarizes the results of a considerable number of experiments in which we investigated the effects of hormones on either (a) the ^{32}P content of phosphatidylinositol in prelabelled hepatocytes or (b) the total phosphatidylinositol content of cells. In

Fig. 2. Time course of angiotensin-stimulated phosphatidylinositol breakdown

Hepatocytes were incubated in ^{32}P -labelled medium for 5 min before the addition of 1 μM -angiotensin and for up to 10 min thereafter. The results plotted are means for triplicate incubations in one of two similar experiments.

most of the experiments a decrease of about 4–8% was seen in the amount or radioactivity of phosphatidylinositol. In the majority of the experiments the replicate assays were sufficiently precise for this small decrease to be statistically significant. There was no change in either the labelling or amount of lipid in the major phospholipids during these incubations (results not shown). The majority of the breakdown experiments were done with vasopressin,

Fig. 3. *Inhibition of angiotensin-stimulated phosphatidylinositol breakdown by [1-sarcosine, 8-isoleucine]angiotensin*. Hepatocytes were incubated with 1 μM-angiotensin for 15 min, together with various amounts of [1-sarcosine, 8-isoleucine]angiotensin. The results plotted are means for triplicate determinations in one of two similar experiments.

since this gave the largest stimulation of phosphatidylinositol labelling, but the limited information on angiotensin and adrenaline suggests that they also provoked phosphatidylinositol breakdown (Table 1).

The role of Ca²⁺ in stimulation of phosphatidylinositol metabolism

We used two different approaches to investigate whether changes in cytosol Ca²⁺ concentration are involved in provoking the phosphatidylinositol responses; first, by admitting Ca²⁺ to cells by using the ionophore A23187, and, secondly, by depleting cells of environmental Ca²⁺.

In experiments with the ionophore, albumin was omitted from the incubation medium to prevent it from being bound in an ineffective form, and the ionophore was presented to the cells 9 min before the addition of Ca²⁺: albumin omission caused a very slight increase in the vasopressin-stimulated incorporation of ³²P into phosphatidylinositol (results not shown). There is abundant evidence that addition of ionophore A23187 to cells at concentrations of 0.5–10 μM is able to effectively activate glycogenolysis (Pointer *et al.*, 1976; Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977) and to inhibit pyruvate kinase (Chan & Exton, 1978). By contrast, these concentrations of ionophore A23187 had only a slight stimulatory effect on the labelling of phosphatidylinositol [the means for two experiments showed rises of 15% at 0.6 μM (not significantly different from control), 45% at 2 μM ($P \sim 0.05$) and 55% at 6 μM ($P < 0.05$)]. In addition, 2 μM-ionophore A23187 had no effect, either stimulatory or inhibitory, on the enhancement of phosphatidylinositol labelling caused by any of the three Ca²⁺-mobilizing hormones.

Table 1. *Breakdown of phosphatidylinositol in hepatocytes exposed to Ca²⁺-mobilizing hormones*

Cells, either (a) ³²P-labelled or (b) unlabelled, were incubated with or without hormones for 15 min and then their phosphatidylinositol content was estimated either radiochemically or by phosphate assay. Results from individual experiments were means $\pm$ s.e.m. for six to 18 incubations; the effects of hormones were then assessed by Student's *t* test. The mean radioactivity or content of phosphatidylinositol in unstimulated cells was in each case set at 100 and the hormone-treated values were expressed as a percentage ($\pm$ s.e.m.) of this value. From this treatment came the presented means derived from the number of experiments indicated in parentheses. Each group of experiments was then examined by the use of a paired *t* test. Abbreviation: n.s., not significant.

	Phosphatidylinositol content or radioactivity (% of control)	Significance of difference from controls for individual experiments by <i>t</i> test	Significance of differences between control and stimulated groups by paired <i>t</i> test
(a) Breakdown of ³²P-labelled phosphatidylinositol			
+ Vasopressin (10 m-units/ml)	94.9 $\pm$ 1.2 (7)	$P < 0.01$ (3), $P < 0.05$ (3), n.s. (1)	$P \sim 0.01$
+ Angiotensin (1 μM)	97.2 $\pm$ 0.3 (4)	$P < 0.05$ (2), n.s. (2)	$P < 0.01$
+ Adrenaline (100 μM)	94.9 $\pm$ 1.6 (4)	$P < 0.01$ (3), n.s. (1)	n.s.
(b) Decrease in phosphatidylinositol content			
+ Vasopressin (10 m-units/ml)	95.1 $\pm$ 0.6 (5)	$P < 0.01$ (3), $P < 0.05$ (1), n.s. (1)	$P < 0.01$

These results suggested that changes in cytosol Ca^{2+} concentration probably play no essential role in the stimulation of phosphatidylinositol metabolism by these hormones. This point of view gained further support from the observation that, as with phosphatidylinositol labelling, the addition of $2\mu\text{M}$ -ionophore A23187 did not either mimic or interfere with vasopressin-stimulated breakdown of phosphatidylinositol. In two independent experiments, the mean phosphatidylinositol content of ionophore A23187-treated cells was 100.4% of the control value (no significant difference), whereas the cells treated with vasopressin (94.5% of control) or with vasopressin and ionophore A23187 (93.7%) were both significantly different from the control value ($P < 0.01$), but not from each other.

Results from the Ca^{2+} -deprivation experiments were somewhat less clear-cut. Cells were isolated in a nominal Ca^{2+} -free medium and each batch of cells was then divided, one batch being washed in buffer containing 0.2mM-EGTA and the other in buffer with 1mM- Ca^{2+} . When stimulated with vasopressin, angiotensin or adrenaline the Ca^{2+} -depleted cells still responded with an increase in phosphatidylinositol labelling, but the magnitude of this stimulation was considerably less than in Ca^{2+} -replete cells (Table 2). In our hands, unstimulated cells incubated in Ca^{2+} -free medium showed greater labelling of phosphatidylinositol than did those incubated with Ca^{2+} , so that the decrease in the hormone-stimulated labelling was clearly apparent both in absolute terms and by comparison with the appropriate controls. This pattern differs somewhat from that reported by Kirk *et al.* (1978), who found decreased labelling in unstimulated Ca^{2+} -free cells, but we do not know why.

Vasopressin-stimulated phosphatidylinositol breakdown appeared still to occur in Ca^{2+} -depleted cells (Table 3), as was expected from the observation that at least some of the vasopressin-stimulated phosphatidylinositol labelling persisted in these cells. In view of the difficulty of measuring the small decrease in cellular phosphatidylinositol content

that was produced by hormonal stimulation, we could not be sure whether the magnitude of the hormone-stimulated phosphatidylinositol breakdown was unchanged after Ca^{2+} -deprivation. An unexpected feature of these experiments was that the phosphatidylinositol content of Ca^{2+} -depleted cells appeared always to be appreciably higher than that of Ca^{2+} -replete cells, irrespective of whether they had been exposed to vasopressin (results not shown). The most likely explanation of this observation appears to be trivial, namely that the absence of Ca^{2+} from the medium in which cells were washed before incubation changed the way in which they packed during centrifugation, but we have not completely ruled out more important effects of Ca^{2+} -depletion either on the phosphatidylinositol content of the cells or on the efficiency of our lipid extraction procedure.

The ligand specificity of the vasopressin receptor

The most active known vasopressin analogue, at least in terms of its antidiuretic potency, is [1-

Table 2. *Effect of Ca^{2+} -deprivation on stimulated labelling of phosphatidylinositol*

Hepatocytes were incubated in ^{32}P -labelled Ca^{2+} -containing or Ca^{2+} -free media for 15min with or without hormones. Results are expressed as means $\pm$ S.E.M. for triplicate incubations in one of two experiments that gave similar results.

	Radioactivity in phosphatidylinositol (d.p.m.)	
	1mM- Ca^{2+}	0.2mM-EGTA
No additions	6920 $\pm$ 580	8590 $\pm$ 900
+ Angiotensin (1 $\mu\text{g}/\text{ml}$)	23900 $\pm$ 90	12340 $\pm$ 570
+ Vasopressin (10m-units/ml)	37810 $\pm$ 610	19600 $\pm$ 360
+ Adrenaline (100 μM)	29290 $\pm$ 850	13590 $\pm$ 1130

Table 3. *Vasopressin-stimulated phosphatidylinositol breakdown in cells deprived of extracellular Ca^{2+}*

Cells were incubated in Ca^{2+} -free conditions as described in the text. Methods and presentation are otherwise as described in Table 1. For statistical analysis, each vasopressin-treated group was compared with the otherwise identical control group containing Ca^{2+} or EGTA. Abbreviation used: n.s., not significant.

	Phosphatidylinositol content (% of Ca^{2+} -containing control)	Significance of difference from appropriate control for individual experiments by <i>t</i> test	Significance of difference between control and stimulated groups by paired <i>t</i> test
1mM- Ca^{2+} + 10m-units of vasopressin/ml	94.4 $\pm$ 1.0 (3)	$P < 0.005$ (2) $P < 0.05$ (1)	$P < 0.05$
0.2mM-EGTA	107 $\pm$ 3.5 (3)		
0.2mM-EGTA + 10m-units of vasopressin/ml	98.5 $\pm$ 0.2 (3)	$P < 0.001$ (2) n.s. (1)	n.s.

deaminocysteine, 2-phenylalanine, 7-(3,4-didehydroproline), 8-arginine] vasopressin, which is about 26 times more potent than [8-arginine] vasopressin (Smith & Walter, 1978). This synthetic molecule was designed to interact with maximum efficiency with the adenylate cyclase-coupled vasopressin receptor of kidney, so it seemed possible that it might discriminate against a Ca^{2+} -mobilizing vasopressin receptor. This was one of the vasopressin analogues tested by Kirk *et al.* (1979), and they indeed found it to be a partial agonist of low efficacy when used to stimulate phosphatidylinositol metabolism. We have obtained independent confirmation of this result. In our hands this analogue produced no appreciable effect on phosphatidylinositol metabolism at concentrations of less than approx. 50–100 nM, and even at 1–10 μM it only produced a 2–4-fold increase in phosphatidylinositol labelling.

Discussion

As a result of the studies reported in the present paper and by others (De Torrentegui & Berthet, 1966; Kirk *et al.*, 1977, 1978), it is clear that phosphatidylinositol metabolism in rat hepatocytes can be stimulated by activation of at least three types of receptor, namely those sensitive to α -adrenergic stimuli, to vasopressin and to angiotensin, but that it is not affected by activation of glucagon receptors (Kirk *et al.*, 1977). The three effective types of receptor all share an ability to activate glycogenolysis by a mechanism that does not involve cyclic AMP and in which an increase in cytosol Ca^{2+} concentration appears to be a key intracellular effector of the hormone effects (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Van de Werve *et al.*, 1977; Hems *et al.*, 1978; Fain, 1978). Thus these results fulfilled our expectation, based on the idea that phosphatidylinositol breakdown might be a reaction essential to all situations involving receptor-controlled Ca^{2+} mobilization, that these three receptors in the hepatocyte should all cause activation of phosphatidylinositol metabolism (see the introduction).

In addition, the unusual ligand selectivity of the vasopressin receptors responsible for initiating phosphatidylinositol breakdown (Kirk *et al.*, 1979; the present study) suggests that these receptors differ markedly from the vasopressin receptors in kidney, which control adenylate cyclase and thus inhibit diuresis. In some other situations in which small ligands (e.g. adrenaline, histamine) exert control over their target cells in these two distinct ways it has become clear this is achieved by the existence of two independent families of receptors, with one controlling adenylate cyclase (e.g. β -adrenergic and H_2 -histaminergic receptors) and the other

controlling Ca^{2+} mobilization (e.g. α -adrenergic and H_1 -histaminergic receptors) (see Michell, 1979). The simplest interpretation of the present observations is to suggest that vasopressin, like catecholamines and histamine (see Michell, 1979), can interact with two different classes of cellular receptors, one of them coupled to adenylate cyclase and the other to a Ca^{2+} -mobilizing mechanism. Although there is already pharmacological evidence that demonstrates that cells can possess more than one type of receptor responsive to the same peptide ligand (e.g. for opioid peptides; Lord *et al.*, 1977), vasopressin appears to be the first peptide ligand for which one can also identify the type of cellular control that is achieved by each class of receptor.

In other systems that have been studied, the accumulated evidence supports the idea that hormone-stimulated phosphatidylinositol metabolism is always initiated through phosphatidylinositol breakdown (Michell *et al.*, 1977a; Jones *et al.*, 1979), and the experiments reported in the present paper indicate that this reaction also initiates the increase in phosphatidylinositol metabolism that occurs in hepatocytes exposed to any of the Ca^{2+} -mobilizing glycogenolytic hormones.

There seems little doubt that removal of Ca^{2+} from the medium surrounding hepatocytes for any appreciable time can prevent most or all glycogenolytic responses to vasopressin and angiotensin, and probably also to adrenaline (e.g. Stubbs *et al.*, 1976; Keppens *et al.*, 1977), even though a response can still be evoked by these hormones in cells in which Ca^{2+} withdrawal occurs only just before addition of any of these hormones (Blackmore *et al.*, 1978). In addition, the glycogenolytic effects of these hormones can be mimicked by using ionophore A23187, a bivalent-cation ionophore, to admit Ca^{2+} to the cells (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977) or to mobilize Ca^{2+} from intracellular stores (Pointer *et al.*, 1976). This suggests that an increase in the cytosol concentration of free Ca^{2+} is alone sufficient to activate glycogenolysis, probably through direct activation of phosphorylase kinase by Ca^{2+} (Vandenheede *et al.*, 1977).

However, the evidence accumulated in the present studies and by Kirk *et al.* (1978) points clearly to the opposite conclusion for hormone-stimulated breakdown of phosphatidylinositol and for hormone-stimulated phosphatidylinositol labelling: these events seem not to be markedly influenced by changes in cytosol Ca^{2+} concentration. Perhaps the most convincing of the items amongst this evidence is the inability of the ionophore A23187 to have any appreciable effect on phosphatidylinositol metabolism: it did not produce phosphatidylinositol breakdown or a substantial stimulation of labelling, nor did it change the responses of these two parameters to activation by hormones. This type of situation, in

which stimulated phosphatidylinositol metabolism is found to be a Ca^{2+} -insensitive response to receptors whose physiological effects are mediated through Ca^{2+} , is now familiar (Trifaró, 1969; Jones & Michell, 1975, 1976, 1978b; Oron *et al.*, 1975; Abdel-Latif, 1976; Bicknell *et al.*, 1979; Fain & Berridge, 1978, 1979; Berridge & Fain, 1979; Jones *et al.*, 1979) and it was this type of observation that originally provoked the suggestion that phosphatidylinositol breakdown might be a reaction essential to stimulus-response coupling at such receptors (Michell, 1975, 1979; Michell *et al.*, 1977a,b; Jones & Michell, 1978b).

Although the simplest interpretation of the previous information on the control of glycogenolysis by Ca^{2+} -mobilizing hormones was to suggest that the main change brought about by activation of appropriate hormone receptors is an increase in cell-surface Ca^{2+} permeability (e.g. Keppens *et al.*, 1977), there is more recent information that may point to a more complex situation. In particular, Chen *et al.* (1978) and Blackmore *et al.* (1978) have both found that there is a rapid net efflux of Ca^{2+} from cells activated by the Ca^{2+} -mobilizing hormones, suggesting initiation by these hormones of the mobilization of Ca^{2+} ions from some intracellular pool, maybe in mitochondria. This would of course require the existence of some intermediary signal to carry the hormone-activation message to the site within the cell from which Ca^{2+} was to be mobilized.

Our original suggestion relating to the function to receptor-stimulated phosphatidylinositol breakdown was that it produced inositol cyclic phosphate (Michell & Lapetina, 1972; Lapetina & Michell, 1973), and it is conceivable that this could play a messenger role in a pathway leading to intracellular Ca^{2+} mobilization. However, no clear evidence has yet been forthcoming that this phosphate ester has an essential role as a messenger at any of the receptors that control phosphatidylinositol breakdown (Michell, 1975; Freinkel & Dawson, 1973; Lapetina & Zieher, 1976), and this has led us more recently to emphasize possible ways in which phosphatidylinositol breakdown might control the permeability of the plasma membrane more directly (Michell *et al.*, 1977a). There is sufficient uncertainty about Ca^{2+} mobilization in the hepatocyte for either type of role for phosphatidylinositol breakdown to be considered seriously. If phosphatidylinositol breakdown is the receptor-coupled initiating event in Ca^{2+} mobilization (in either manner) then it must occur at the plasma membrane; the cellular site at which this reaction occurs is therefore the next essential point that must be established.

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