Biochem. J. (1975) **148**, 479–485
Printed in Great Britain

The Relationship of Calcium to Receptor-Controlled Stimulation of Phosphatidylinositol Turnover

EFFECTS OF ACETYLCHOLINE, ADRENALINE, CALCIUM IONS, CINCHOCAINE AND A BIVALENT CATION IONOPHORE ON RAT PAROTID-GLAND FRAGMENTS

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(*Received* 31 *January* 1975)

1. The possibility that Ca^{2+} ions are involved in the control of the increased phosphatidylinositol turnover which is provoked by α-adrenergic or muscarinic cholinergic stimulation of rat parotid-gland fragments has been investigated. Both types of stimulation provoked phosphatidylinositol breakdown, which was detected either chemically or radiochemically, and provoked a compensatory synthesis of the lipid, detected as an increased rate of incorporation of $^{32}P_i$ into phosphatidylinositol. 2. Acetylcholine had little effect on the incorporation of labelled glycerol, whereas adrenaline stimulated it significantly, but to a much lower extent than ^{32}P incorporation: this suggests that the response to acetylcholine was entirely accounted for by renewal of the phosphorylinositol head-group of the lipid, but that some synthesis *de novo* was involved in the response to adrenaline. 3. The responses to both types of stimulation, whether measured as phosphatidylinositol breakdown or as phosphatidylinositol labelling, occurred equally well in incubation media containing 2.5mM-Ca^{2+} or 0.2mM-EGTA [ethanedioxybis(ethylamine)-tetra-acetic acid]. 4. Incubation with a bivalent cation ionophore (A23187) led to a small and more variable increase in phosphatidylinositol labelling with $^{32}P_i$, which occurred whether or not Ca^{2+} was available in the extracellular medium: this was not accompanied by significant phosphatidylinositol breakdown. 5. Cinchocaine, a local anaesthetic, produced parallel increases in the incorporation of P_i and glycerol into phosphatidylinositol. This is compatible with its known ability to inhibit phosphatidate phosphohydrolase (EC 3.1.3.4) and increase phosphatidylinositol synthesis *de novo* in other cells. 6. These results indicate that the phosphatidylinositol turnover evoked by α-adrenergic or muscarinic cholinergic stimuli in rat parotid gland probably does not depend on an influx of Ca^{2+} into the cells in response to stimulation. This is in marked contrast with the K^+ efflux from this tissue, which is controlled by the same receptors, but is strictly dependent on the presence of extracellular Ca^{2+}. The Ca^{2+}-independence of stimulated phosphatidylinositol metabolism may mean that it is controlled through a mode of receptor function different from that which controls other cell responses. Alternatively, it can be interpreted as indicating that stimulated phosphatidylinositol breakdown is intimately involved in the mechanisms of action of α-adrenergic and muscarinic cholinergic receptor systems.

The increased phosphatidylinositol turnover which occurs in many tissues when exposed to appropriate extracellular stimuli is probably a result of an increase in the rate of removal of the phosphorylinositol group of the lipid [see Michell (1975) for review]. In support of this view, it has been demonstrated that acetylcholine and pancreozymin cause a decrease in the phosphatidylinositol content of mouse pancreas (Hokin-Neaverson, 1974*a*,*b*) and that acetylcholine has the same effect on rat parotid-gland fragments (Jones & Michell, 1974). In the present paper it is shown that adrenergic stimulation of rat parotid-gland fragments causes a similar effect. Possible functions of this phosphatidylinositol breakdown include the generation of changes in the phosphatidylinositol concentration of the plasma membrane or the release of *myo*-inositol 1:2-cyclic phosphate within the cells.

The function of this response cannot be defined with any certainty until it is known exactly how the interaction of an extracellular ligand with a cell-surface receptor site leads to its initiation. It is usually provoked by stimuli which do not stimulate adenylate cyclase or elevate intracellular cyclic AMP; it therefore seems certain that cyclic AMP cannot be the mediator of this response (Michell, 1975). On the

other hand, there is quite a high positive correlation between the effects of various stimuli in increasing the flux of Ca^{2+} across the cell surface, elevating the intracellular concentration of cyclic GMP, and stimulating phosphatidylinositol turnover (Michell, 1975). As it seems likely that Ca^{2+} fluxes precede the effects of acetylcholine on cyclic GMP (Schultz *et al.*, 1973) it appeared necessary to determine whether Ca^{2+} is responsible for triggering phosphatidylinositol turnover. Previous studies have suggested, on the basis solely of comparisons of incubations with and without Ca^{2+}, that enhanced phosphatidylinositol labelling might be independent of Ca^{2+} (Hokin, 1966; Trifaró, 1969). The present paper extends such studies to include measurements of both breakdown and synthesis of phosphatidylinositol and also experiments in which a bivalent cation ionophore and a local anaesthetic were used to modify Ca^{2+} distribution at cell membranes.

Materials and Methods

Most materials were from sources described previously (Michell & Jones, 1974; Jones & Michell, 1974; Allan & Michell, 1974*a*). The antibiotic A23187 [the structure for which is given by Chaney *et al.* (1974)] was a gift from the Lilly Research Centre, Windlesham, Surrey GU20 6PH, U.K.

The methods for tissue preparation, incubation and lipid analysis were largely those used previously (Michell & Jones, 1974; Jones & Michell, 1974). Incubations were for 30min. When the rate of incorporation of either [2-^{3}H]glycerol or [1,3-^{3}H]-glycerol into lipids was to be measured, the labelled compound was added to incubation media to a concentration of 1.0μCi/ml (420μCi/μmol) or 0.5μCi/ml (2.1mCi/μmol) respectively. The lipids were separated by chromatography as described previously (Jones & Michell, 1974), but were detected by exposure to iodine vapour and their radioactivity was determined by liquid-scintillation counting (Allan & Michell, 1975).

The results are presented in the form of ratios which compare phosphatidylinositol with a mixed lipid sample containing most of the tissue phospholipids. Conclusions based on such a method are only reliable if the properties of this latter material either remain unchanged or change slightly in the same direction as phosphatidylinositol. The validity of this assumption for $^{32}P_i$ incorporation has previously been demonstrated by us for adrenergic and cholinergic stimuli (Michell & Jones, 1974; Jones & Michell, 1974) and we have also shown it to be correct for cinchocaine and compound A23187 (unpublished work). Specific radioactivities were not determined in the experiments with labelled glycerol, but the total incorporation into the major phospholipid spot, in a series of similar tissue samples, was not decreased by any of the drugs tested at the concentrations used in these studies. Hence the expression of the results in this form gives either a correct or slightly underestimated measure of any specific effects on phosphatidylinositol metabolism.

Results

In general, four types of information were sought in the study of the effects of each of the drugs that were investigated. First, the effect on incorporation of $^{32}P_i$ into phosphatidylinositol was measured. A positive response in this assay indicates either an increased rate of turnover of the phosphorylinositol group of phosphatidylinositol, or an increase in the rate of biosynthesis of phosphatidylinositol *de novo*. Increased turnover is provoked by many agents which stimulate cells through interactions with cell-surface receptor mechanisms (see Michell, 1975), and increased synthesis *de novo* can be a response to amphiphilic cationic drugs (Allan & Michell, 1975; Brindley *et al.*, 1975). The second and third types of measurement, namely measurements of any changes in tissue phosphatidylinositol concentration and of the rate of incorporation of labelled glycerol into phosphatidylinositol, were designed to distinguish between these two possibilities. If a drug were to produce only turnover of the phosphorylinositol group triggered by an increased rate of phosphatidylinositol breakdown, then there should be a fall in tissue phosphatidylinositol concentration and no increase in the rate of glycerol incorporation. If the response were to consist of an increase in synthesis *de novo*, there should be no fall in concentration and a large rise in the incorporation of labelled glycerol. Finally, the effects of these responses on the omission of Ca^{2+} from the incubation medium were determined.

Effects of Ca^{2+} on phosphatidylinositol labelling

Increased phosphatidylinositol labelling was consistently observed in tissue incubated in Ca^{2+}-free media, as compared with control media, in both control incubations and in tissue treated with acetylcholine, adrenaline or compound A23187 (Tables 1, 3 and 6). This effect may have been due to an increased rate of entry of P_i into the cells, a change in the size or the specific radioactivity of an intracellular pool of some precursor of phosphatidylinositol, or a slight decrease in the incorporation of $^{32}P_i$ into other phospholipids.

Effects of acetylcholine

Muscarinic cholinergic stimulation of parotid-gland fragments produced a marked breakdown of

Table 1. *Effects of acetylcholine on the labelling of phosphatidylinositol from $^{32}P_i$ and on phosphatidylinositol concentration in parotid-gland fragments in the presence and absence of extracellular Ca^{2+}*

Tissue fragments from six rats were pooled, divided into two portions, and incubated in ^{32}P-labelled Krebs–Ringer bicarbonate medium containing 5mM-3-hydroxybutyrate and either 2.5mM-Ca^{2+} and 0.2mM-EGTA (or EGTA alone) for 60min. The fragments were collected, divided into samples and incubated for a further 30min in similar ^{32}P-containing media, to which acetylcholine (10μM) and eserine (100μM) were added as indicated. Results are means±S.E.M. (numbers of tissue samples analysed).

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of phosphate contents (phosphatidylinositol/other phospholipids)
Ca^{2+}	0.32±0.001 (8)	0.133±0.007 (8)
Ca^{2+} + acetylcholine and eserine	1.50±0.010 (8)*	0.090±0.011 (9)*
EGTA	0.52±0.002 (8)	0.142±0.007 (7)
EGTA + acetylcholine and eserine	2.17±0.006 (8)*	0.110±0.004 (8)*

* Significantly different from equivalent incubations without acetylcholine ($P \leqslant 0.001$).

Table 2. *Effects of acetylcholine on phosphatidylinositol labelling from $^{32}P_i$ and from [2-^3H]glycerol in parotid-gland fragments*

Experimental details and expression of results were as described in Table 1 except that in some incubations [2-^{3}H]glycerol was used instead of $^{32}P_i$. Ca^{2+} (2.5mM) was present throughout.

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of [^{3}H]glycerol incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)
No additions	0.40±0.02 (5)	0.092±0.005 (9)
+ Acetylcholine and eserine	1.29±0.01 (5)*	0.111±0.008 (10)†

* Significantly different from control ($P<0.001$).
† No significant difference from control ($P\sim0.1$).

phosphatidylinositol, detected as either a decrease in phosphatidylinositol concentration, or a loss of ^{32}P from pre-labelled lipid (Table 1 and Jones & Michell, 1974). It also produced a large increase in the incorporation of $^{32}P_i$ into phosphatidylinositol (Table 1). These changes were not accompanied by any appreciable change in the rate of incorporation of labelled glycerol (Table 2), and it therefore appears that this response consists of an increase in the turnover of the phosphatidylinositol head-group.

When Ca^{2+} was omitted from the incubation medium and EGTA included there was no decrease in the effects of acetylcholine on either phosphatidylinositol breakdown or its labelling from $^{32}P_i$ (Table 1).

Effects of adrenaline

It has previously been demonstrated that α-adrenergic stimuli produce increased incorporation of $^{32}P_i$ into phosphatidylinositol in parotid-gland fragments (Oron *et al.*, 1973; Michell & Jones, 1974). Table 3 indicates that this response also involves phosphatidylinositol breakdown, although the magnitude of the fall in phosphatidylinositol concentration was smaller than with acetylcholine. In response to adrenaline there was a significant rise in the labelling of phosphatidylinositol with [^{3}H]glycerol, although the effect on glycerol incorporation was much smaller than the increase in incorporation of $^{32}P_i$ (Table 4). These observations suggest that much of the phosphatidylinositol synthesized in response to adrenaline re-utilizes diacylglycerol released during phosphatidylinositol breakdown, but that there is also a contribution from synthesis *de novo*.

As with acetylcholine, the stimulations by adrenaline of both breakdown and synthesis of phosphatidylinositol occurred whether the incubation media contained Ca^{2+} or EGTA (Table 3).

Effects of cinchocaine and other amphiphilic cations

In lymphocytes, and probably in several other tissues, amphiphilic cationic drugs cause an inhibition of phosphatidate phosphohydrolase which leads to redirection of newly synthesized phosphatidate to the synthesis *de novo* of phosphatidylinositol (Allan & Michell, 1975; Brindley *et al.*, 1975). It seems that a

Table 3. *Effects of adrenaline on labelling of phosphatidylinositol from $^{32}P_i$ and on phosphatidylinositol concentration in parotid-gland fragments in the presence and absence of extracellular Ca^{2+}*

Experimental conditions and expression of results were essentially as in Table 1 except that the drug used was adrenaline (100 μM), and EGTA was not present in the incubations which contained Ca^{2+}. The information for tissue in the absence of Ca^{2+} was derived from tissue samples in which the 'unstimulated' phosphatidylinositol concentration was atypically low: the reason for this is not known.

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of phosphate contents (phosphatidylinositol/other phospholipids)
Ca^{2+}	0.57±0.05 (14)	0.113±0.003 (21)
Ca^{2+}+adrenaline	1.41±0.13 (7)*	0.090±0.004 (18)*
EGTA	0.81±0.03 (18)	0.087±0.002 (13)
EGTA+adrenaline	2.61±0.17 (19)*	0.067±0.003 (13)*

* Significantly different from incubations without adrenaline ($P<0.001$).

Table 4. *Effects of adrenaline on phosphatidylinositol labelling from $^{32}P_i$ and from [2-^{3}H]glycerol in parotid-gland fragments*

Experimental details were as in Table 2, except that the drug used was adrenaline (100 μM). Expression of results is as in Table 1.

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of [^{3}H]glycerol incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)
No additions	0.32±0.01 (5)	0.166±0.008 (5)
+Adrenaline	1.42±0.05 (5)*	0.312±0.016 (5)*

* Significantly different from incubations without adrenaline ($P<0.001$).

Table 5. *Effects of cinchocaine on labelling of phosphatidylinositol and on phosphatidylinositol concentration in parotid-gland fragments*

Conditions were as in Tables 1 and 2, except that the drug used was cinchocaine (0.5 mM); 2.5 mM-Ca^{2+} was present throughout. Expression of results is as in Table 1.

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of [^{3}H]glycerol incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of phosphate contents (phosphatidylinositol/other phospholipids)
No additions	0.54±0.01 (5)	0.102±0.010 (4)	0.071±0.012 (4)
+Cinchocaine	2.05±0.09 (5)*	0.57 ±0.05 (4)*	0.092±0.009 (5)†

* Significantly different from incubations without cinchocaine ($P<0.001$).
† No significant difference from incubations without cinchocaine ($P\sim0.3$).

similar situation applies in the parotid gland in that cinchocaine, a potent local anaesthetic, stimulated the incorporation of $^{32}P_i$ and of labelled glycerol into phosphatidylinositol to similar extents without a significant change in the concentration of this lipid (Table 5). Similar effects on incorporation of $^{32}P_i$ into the phosphatidylinositol of parotid fragments were produced by three other amphiphilic cationic drugs, namely chlorpromazine, trifluoperazine and propranolol (results not shown).

Effects of compound A23187

Compound A23187 is a lipophilic antibiotic which binds Ca^{2+}, Mg^{2+} and a variety of other multivalent cations and which catalyses the movement of these ions across lipid or lipoprotein barriers such as biological membranes (Pfeiffer *et al.*, 1974). One application of this compound is in testing the role in receptor phenomena of Ca^{2+} influxes into cells exposed to stimuli. In such situations compound

A23187 can be used to directly facilitate the entry of Ca^{2+} into cells and therefore to distinguish between responses which are secondary to the increased Ca^{2+} influx and those which do not depend on the occurrence of such a Ca^{2+} influx.

The effects of compound A23187 on parotid phospholipid metabolism were neither as large nor as consistent as the effects of the neurotransmitters and amphiphilic amines. An increase in incorporation of $^{32}P_i$ into phosphatidylinositol was observed consistently (Tables 6, 7*a* and 7*b*); that this represented a real increase specific to phosphatidylinositol was confirmed by the observation that no significant change was seen in the specific radioactivity of the major phospholipid spot in these experiments (results not shown). In one experiment, the increased incorporation of $^{32}P_i$ into phosphatidylinositol was accompanied by an increase in the incorporation of labelled glycerol (Table 7*a*), but in other experiments no such effect was seen (Table 7*b*); the reason for this variation is not known.

Compound A23187 did not provoke any significant change in the phosphatidylinositol content of parotid fragments and its effects on incorporation of $^{32}P_i$ into phosphatidylinositol did not depend on the availability of extracellular Ca^{2+} (Table 6).

Discussion

The enzymic mechanism underlying enhanced phosphatidylinositol turnover has been thought for some time to consist of a cycle of four reactions in which the phosphorylinositol group of phosphatidylinositol is removed and renewed, with the whole reaction sequence achieving conservation of the diacylglycerol portion of the molecule [see Michell

Table 6. *Effects of a bivalent cation ionophore (A23187) on labelling of phosphatidylinositol and on phosphatidylinositol concentration in parotid-gland fragments in the presence and absence of extracellular* Ca^{2+}

Conditions were as in Tables 1 and 2, except that the drug used was compound A23187 (10 μg/ml). This was added as a stock solution in methanol (1000 μg/ml) and an appropriate quantity of methanol was added to incubations not containing compound A23187: this amount of methanol had no significant effect on the quantities being measured. Expression of results is as in Table 1.

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of phosphate content (phosphatidylinositol/other phospholipids)
Ca^{2+}	0.37 ± 0.001 (21)	0.131 ± 0.005 (23)
Ca^{2+} + compound A23187	0.79 ± 0.003 (19)*	0.115 ± 0.004 (19)†
EGTA	0.69 ± 0.003 (9)	0.153 ± 0.012 (9)
EGTA + compound A23187	1.05 ± 0.006 (9)*	0.139 ± 0.014 (8)†

* Significantly different from value without compound A23187 ($P < 0.001$).
† No significant difference from value without compound A23187 ($P \geqslant 0.1$).

Table 7. *Effects of compound A23187 on phosphatidylinositol labelling from* $^{32}P_i$ *and from labelled glycerol in parotid-gland fragments*

Experimental details were as in Table 2 except that the drug used was compound A23187 (10 μg/ml; see legend to Table 6) and that [1,3-^{3}H]glycerol was used to obtain the values under (*b*). Expression of results is as in Table 1.

	Ratio of $^{32}P_i$ incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)	Ratio of [^{3}H]glycerol incorporations (d.p.m. in phosphatidylinositol/d.p.m. in other phospholipids)
(*a*) No additions	0.29 ± 0.01 (5)	0.15 ± 0.01 (5)
+ compound A23187	0.38 ± 0.02 (5)‡	0.20 ± 0.01 (5)§
(*b*) No additions	0.72 ± 0.05 (8)	0.15 ± 0.005 (15)
+ compound A23187	1.13 ± 0.04 (6)*	0.16 ± 0.009 (15)†

* Significantly different from incubations without compound A23187 ($P \leqslant 0.005$).
† Not significantly different from incubations without compound A23187 ($P \sim 0.3$).
‡ Significantly different from incubations without compound A23187 ($0.05 < P < 0.01$)
§ Significantly different from incubations without compound A23187 ($P \sim 0.01$).

(1975) for review]. It was shown that cholinergic stimulation of either pancreas or parotid gland, and pancreozymin treatment of pancreas, caused a fall in tissue phosphatidylinositol concentration, and it was concluded that the stimulated reaction in this cycle was the removal of the head-group from phosphatidylinositol (Hokin-Neaverson, 1974*a*,*b*; Jones & Michell, 1974). Although there are strong arguments for believing that this is also the stimulated reaction in the responses to a variety of other physiological stimuli (see Michell, 1975), the information in Table 1, which shows that tissue phosphatidylinositol concentration falls in response to adrenergic stimulation, is the first unequivocal evidence on this point.

The receptors that control phosphatidylinositol breakdown (and resynthesis) in rat parotid gland are of the α-adrenergic and muscarinic cholinergic types (Oron *et al.*, 1973; Michell & Jones, 1974; Jones & Michell, 1974). It is not known exactly how activation of these receptor systems leads to control of cellular responses in target tissues, but there is considerable evidence that appears to implicate Ca^{2+} and cyclic GMP. In particular, it has been shown that in the rat parotid gland, the organ that we have been studying, activation of either of these receptor systems leads to a rapid efflux of K^+ from the tissue. This response is abolished if Ca^{2+} is withdrawn from the extracellular medium (Selinger *et al.*, 1973) and can be triggered in the absence of either acetylcholine or adrenaline by the addition of compound A23187, a bivalent cation ionophore (Selinger *et al.*, 1974). These results have suggested, at least so far as the triggering of the K^+ efflux is concerned, that the most important effect of activation of α-adrenergic and muscarinic cholinergic receptors is to increase Ca^{2+} influx into cells, and that this intracellular Ca^{2+} provokes the K^+ efflux.

The experiments described in the present paper were designed to determine whether the phosphatidylinositol turnover response to α-adrenergic or muscarinic cholinergic stimulation in this gland is also a result of Ca^{2+} entry into the tissue. In general, they suggest that it is not. First, the responses to adrenaline and acetylcholine, whether measured in terms of phosphatidylinositol breakdown or its synthesis, were both unaffected by the omission of Ca^{2+} from the extracellular medium. The same conclusion was reached in previous studies on the effect of acetylcholine on phosphatidylinositol labelling with $^{32}P_i$ in both pancreas (Hokin, 1966) and adrenal medulla (Trifaró, 1969). Similar results were also obtained with ADP-treated platelets (Lloyd *et al.*, 1973). The only situation where there does appear to be a well-documented Ca^{2+} requirement for a change in phospholipid labelling in response to stimulation is in electrically depolarized synaptosomes (Hawthorne & Bleasdale, 1975); the mechanism of this effect may, however, be fundamentally different, since it does not involve participation of a hormone-sensitive receptor system and phosphatidate is the only lipid affected. There is also preliminary evidence which might indicate such a Ca^{2+} requirement in thyroid-stimulating-hormone-treated thyroid (Zor *et al.*, 1968), although further work is needed on this system.

Second, the effects of compound A23187 on ^{32}P incorporation into phosphatidylinositol, although significant, do not appear to be dependent on the presence of extracellular Ca^{2+}, whereas the triggering of the K^+ efflux from parotid fragments with the same concentration of this agent does depend on a supply of extracellular Ca^{2+} (Selinger *et al.*, 1974). Further, there was no evidence that compound A23187 caused a significant breakdown of phosphatidylinositol. Thus there appears to be no evidence, at least in the parotid gland, that ionophore-mediated Ca^{2+} influxes can trigger the increased phosphatidylinositol turnover which is elicited by physiological stimuli such as neurotransmitters. Recently we (D. Allan & R. H. Michell, unpublished work) have attempted to use lymphocytes, which show enhanced phosphatidylinositol turnover in response to mitogens (see Maino *et al.*, 1975), to test the generality of this conclusion. The results obtained have, however, not provided conclusive evidence either for or against a role for Ca^{2+} in the phosphatidylinositol response of lymphocytes, despite the fact that phosphatidylinositol breakdown in a soluble fraction from lymphocytes has a requirement for Ca^{2+} (Allan & Michell, 1974*b*).

There appear to be at least two reasonable ways to explain why removal of extracellular Ca^{2+} from parotid fragments has no effect on phosphatidylinositol turnover, but abolishes K^+ efflux, even though both responses are elicited by stimulation of the same receptor populations. In one it might be argued that phosphatidylinositol turnover and K^+ efflux are representatives of two types of response which are triggered through different receptor-linked mechanisms: this seems unlikely in view of the wide range of receptor types and tissues which display similar phosphatidylinositol turnover responses. The other alternative, that phosphatidylinositol breakdown is a reaction central to the mechanisms of cell-surface receptor systems, is more attractive, but unproven. If it is correct, then phosphatidylinositol breakdown must be an early step in the reaction sequence involved in these systems, preceding the involvement of Ca^{2+} from extracellular sources. Some possible experimental approaches to resolution of this uncertainty have been reviewed (Michell, 1975).

Note Added in Proof (Received 15 April 1975)

Recent experiments in other laboratories have indicated that the phosphatidylinositol response of the rat parotid gland is independent of extracellular

Ca^{2+} and is not triggered by compound A23187 (Rossignol *et al.*, 1974; Oron *et al.*, 1975). Their interpretations of the significance of these observations were, however, different from ours (see the present paper and Michell, 1975).

We are grateful to the Medical Research Council for financial support and to Dr. D. Allan, Dr. R. Coleman and Dr. J. B. Finean for valuable discussions. Professor J. N. Hawthorne and Dr. M. J. Crumpton kindly made manuscripts available before publication.

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