Breakdown of Phosphatidylinositol Provoked by Muscarinic Cholinergic Stimulation of Rat Parotid-Gland Fragments

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(Received 29 March 1974)

When rat parotid fragments that had been labelled with ^{32}P *in vivo* were exposed to high concentrations of acetylcholine, radioactivity was lost from phosphatidylinositol but not from other phospholipids. Simultaneously the concentration of phosphatidylinositol in the tissue decreased. If previously unlabelled tissue was incubated with $^{32}\text{P}_i$ an increase in incorporation of radioactivity into phosphatidylinositol was observed during this decrease in concentration. The effects of acetylcholine were blocked by atropine, but not by tubocurarine. The response to acetylcholine was rapid, with up to one-third of the tissue's phosphatidylinositol disappearing within 5 min. Similar effects were evoked by stimulation with methacholine and by high concentrations of tetramethylammonium ion; these responses were also atropine-sensitive and tubocurarine-insensitive. It is concluded that the event in inositol lipid metabolism that is affected by acetylcholine stimulation is removal of the phosphorylinositol group from the molecule; this is mediated through muscarinic cholinergic receptors. This is followed by a compensatory increase in the rate of synthesis of phosphatidylinositol, which has been described in detail in the past. These observations are compared with those of previous workers and are discussed in relation to the existing hypotheses relating to the significance of stimulus-provoked phosphatidylinositol turnover.

A specific increase in the rate of incorporation of labelled P_i and inositol into phosphatidylinositol occurs in many tissues when they are exposed to appropriate extracellular stimuli (Hokin, 1968, 1969*a,b*; Lapetina & Michell, 1973*a*; Hawthorne, 1973). Two types of possible mechanisms for this effect, namely changes in the specific radioactivity of intracellular precursor pools and direct enhancement of the biosynthesis of phosphatidylinositol *de novo*, appear to have been excluded by existing experimental data. A third type of mechanism in which the primary event evoked by the stimulus is the removal of the phosphorylinositol group from phosphatidylinositol has been favoured by several authors (Hokin, 1967; Durell *et al.*, 1969; De Robertis, 1971; Michell & Lapetina, 1972; Lapetina & Michell, 1973*a*; Freinkel & Dawson, 1973; Lapetina & Michell, 1974). Further alternatives include stimulation of either diacylglycerol kinase (Hokin & Hokin, 1959; Hollander *et al.*, 1970) or phosphatidate phosphohydrolase (Schacht & Agranoff, 1973; Yagihara *et al.*, 1973).

In the hypothesis which envisages removal of the phosphorylinositol group from phosphatidylinositol as the key step the enhanced incorporation of precursors into phosphatidylinositol is seen as a secondary phenomenon which represents replenishment of the cell's depleted phosphatidylinositol

complement. The whole cycle of removal and resynthesis thus constitutes an enhancement of phosphatidylinositol turnover. Two of the experimental predictions which arise from this model appear to be easily tested. First, if a tissue which contains phosphatidylinositol with a label in its phosphorylinositol group is stimulated then radioactivity should be lost from the lipid. Second, if the rate of phosphatidylinositol synthesis in a stimulated tissue is inadequate to match the rate of phosphatidylinositol cleavage there should be a decrease in the phosphatidylinositol concentration in the tissue. The present paper describes these two effects in acetylcholine-stimulated rat parotid fragments and defines the acetylcholine receptor which is involved in its initiation. Increased phosphatidylinositol labelling in acetylcholine-treated parotid gland has been reported previously by Hokin & Sherwin (1957) and by Eggman & Hokin (1960).

Materials and Methods

Materials

Most materials were from sources previously described (Lapetina & Hawthorne, 1971; Michell & Jones, 1974). Acetyl- β -methylcholine (methacholine), tetramethylammonium chloride and atropine

pine were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and tubocurarine was from Burroughs Wellcome and Co., Temple Hill, Dartford, Kent, U.K.

Methods

Incubations were carried out in a modified Krebs-Ringer bicarbonate medium containing 14mM-K⁺ and 5mM-2-hydroxybutyrate essentially as described previously (Michell & Jones, 1974). Phosphatidylinositol metabolism was measured in three different ways; for this purpose three slightly different types of incubation in this medium were used.

(a) ³²P_i was included in the incubation medium. Tissue fragments were incubated in the radioactive medium for 1h in the absence of agonists. These were then added and incubation continued for a further 30min or 60min. This design, in which the effects of agonists on ³²P_i incorporation into phospholipids is determined, is identical with that used previously (Michell & Jones, 1974).

(b) Four to six rats were each injected with 0.5mCi of ³²P_i and deprived of food 18h before death. The radioactive parotid glands were removed from these animals and incubated in unlabelled medium for 1h. The tissue was recovered and divided into samples; some were homogenized in chloroform-methanol (1:2, v/v) and the remainder incubated for a further period in non-radioactive medium, with or without added agonists. This allowed us to measure the loss of the radioactive head group from phosphatidylinositol which was induced by stimulation.

(c) The third approach was to measure the phosphatidylinositol content of the tissue samples before and after the addition of agonists. This was done either (a) in incubations in which neither the medium nor the slices were radioactive or (b) in conjunction with either of the two preceding approaches, in a way that allowed us to determine whether the stimulated ³²P_i incorporation or the loss of label were accompanied by changes in tissue phosphatidylinositol concentration.

Lipids were extracted from the tissue samples, separated by chromatography on formaldehyde-treated papers, and analysed for phosphate and radioactivity as described by Michell & Jones (1974). The pattern of separation on these papers is such that phosphatidylinositol is well-separated from the other major phospholipids of the tissue (Wagner *et al.*, 1962). In good separations phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine may be resolved (White & Larrabee, 1973), but frequently they are not. As the effects of acetylcholine on phospholipid metabolism in tissue fragments are almost confined to phosphatidylinositol we have analysed only phosphatidylinositol and the whole of the faster-running area from

each of our paper chromatograms; this approach has previously proved valid (Michell & Lapetina, 1972; Lapetina & Michell, 1973b; Michell & Jones, 1974). In our experiments the latter area would normally include phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, diphosphatidylglycerol and a small amount of phosphatidic acid. Results are presented mainly in the form of ratios which compare phosphatidylinositol with this mixed sample which contains almost all of the other phospholipids of the tissue.

Results

Increased incorporation of ³²P_i into phosphatidylinositol in response to acetylcholine

Parotid fragments which had been incubated with ³²P_i for 60min were exposed to acetylcholine and eserine during a further 30 or 60min incubation in radioactive medium. The rate of incorporation of ³²P into phosphatidylinositol was greatly increased by this treatment; the other phospholipids were much less highly labelled and showed little response (Table 1).

Loss of radioactive phosphatidylinositol and decrease in phosphatidylinositol concentration in response to acetylcholine

When slices which had been labelled with ³²P *in vivo* were exposed to acetylcholine *in vitro* there was a marked loss of radioactivity from phosphatidylinositol (Table 2); there was no loss in slices incubated without acetylcholine. Simultaneously there was an approximately equivalent fall in the tissue phosphatidylinositol concentration (Table 2); again the effect was only observed in the presence of acetylcholine. Both the radioactivity and the phosphorus content of the area containing other

Table 1. *Effect of acetylcholine on ³²P_i incorporation into phosphatidylinositol and into other phospholipids*

Parotid fragments were incubated for 60min in the absence of acetylcholine. Acetylcholine (10μM in Expt. 1, 100μM in Expt. 2) and eserine (100μM) were added and incubation was continued for a further 30min (Expt. 1) or 60min (Expt. 2). Results are means ± S.E.M. and the number of incubations analysed is given in parentheses.

	Specific radioactivities (c.p.m. of ³² P/nmol of P)			
	Phosphatidylinositol		Other phospholipids	
	No additions	+Acetylcholine and eserine	No additions	+Acetylcholine and eserine
Expt. 1	60 ± 6 (8)	277 ± 14 (9)	14 ± 2 (9)	27 ± 1 (9)
Expt. 2	59 ± 2 (3)	422 ± 37 (5)	11 ± 2 (5)	19 ± 1 (4)

Table 2. *Acetylcholine-induced loss of radioactivity from phosphatidylinositol and decrease in phosphatidylinositol concentration*

Parotid-gland fragments obtained from ^{32}P -labelled rats were incubated in unlabelled media for 60 min. Some samples were taken for analysis ('zero time') and the remaining samples were incubated in fresh unlabelled media for a further 30 min either with or without acetylcholine ($10\mu\text{M}$) and eserine ($100\mu\text{M}$). Results are given as the mean $\pm$ S.E.M. (number of incubations analysed).

	Ratios of	
	Radioactivity (c.p.m. in phosphatidylinositol/ c.p.m. in other phospholipids)	Phosphate content (phosphatidylinositol/ other phospholipids)
Zero time	0.148 ± 0.005 (10)	0.120 ± 0.005 (23)
Incubated 30min	0.153 ± 0.007 (10)	0.111 ± 0.003 (20)
Incubated 30min with acetylcholine and eserine	0.101 ± 0.004 (10)*	0.082 ± 0.003 (22)*
Percentage decrease:		
Relative to zero-time	32	32
Relative to 30min incubation	34	26

* Significant difference from zero time ($P < 0.001$).

phospholipids were unchanged by acetylcholine treatment, as, in separate experiments, was the total phospholipid content of the tissue (per g wet weight).

The rate at which the tissue phosphatidylinositol concentration fell in response to acetylcholine was a function of the acetylcholine concentration until, in the presence of 2mM -acetylcholine, about one-third of the total was lost in only 5 min (Fig. 1). The maximum extent of the fall was variable; usually it was about 30%, but values close to zero and above 50% have been observed. One reason for this scatter may be some degree of variability in the ability of different tissue preparations to synthesize phosphatidylinositol to replace that which they have lost. This idea is favoured by the observation that slices which are incubated in media containing factors required for the synthesis of phosphatidylinositol (inositol and/or cytidine) usually show a smaller fall in phosphatidylinositol concentration and a larger stimulation of $^{32}\text{P}_i$ incorporation into phosphatidylinositol than those incubated in their absence (L. M. Jones & R. H. Michell, unpublished work).

In some experiments both $^{32}\text{P}_i$ incorporation and phosphatidylinositol concentration were measured in the same tissue samples: enhanced phosphatidylinositol breakdown and synthesis could both be observed simultaneously (Table 3, and see also Tables 5 and 6).

Effects of nicotinic-receptor and muscarinic-receptor blockade

Acetylcholine receptors are of two general types. Nicotinic receptors mediate transmission through sympathetic ganglia and the response of

skeletal muscle to nervous stimulation, whereas one of the best-characterized responses to muscarinic stimulation is smooth-muscle contraction. Tubocurarine blocks the action of acetylcholine at nicotinic receptors, and atropine has an equivalent effect at muscarinic sites. In our experiments we have only observed prevention of the effects of acetylcholine by atropine (Table 4); tubocurarine was without effect on the acetylcholine-induced decrease in tissue phosphatidylinositol concentration. Atropine, in fact, not only prevented the acetylcholine-induced loss of phosphatidylinositol, but also appeared to elicit a slight increase in its labelling. This seems likely to have been a result of its ability, by blocking muscarinic receptors, to prevent even the small degree of breakdown of newly synthesized phosphatidylinositol that would have occurred in unstimulated tissue fragments as a part of the normal phosphatidylinositol turnover of resting tissue.

Effects of stimuli specific for muscarinic or nicotinic receptors

Methacholine (acetyl- β -methylcholine) is a muscarinic cholinomimetic agent slightly less potent than acetylcholine. It is effective in evoking effects on phosphatidylinositol metabolism similar to those produced by acetylcholine; these effects are atropine-sensitive (Table 5).

On the other hand, tetramethylammonium acts mainly at nicotinic receptors and is much less potent than acetylcholine. It had little effect in our experiments except at a high concentration (Table 6); at this concentration it provoked, like acetylcholine, a decrease in tissue phosphatidylinositol which was accompanied by an increased incorporation of $^{32}\text{P}_i$

Fig. 1. Decrease in phosphatidylinositol content of rat parotid fragments in response to acetylcholine

Unlabelled tissue fragments were incubated for 60 min in unlabelled media. They were then divided into samples and incubated for a further period in the absence or presence of eserine (usually 100 μM) and the specified concentration of acetylcholine. Results are expressed as mean $\pm$ S.E.M. of the number of incubations given in parentheses: (a) 10 μM-acetylcholine: Δ , control (12) and $\blacktriangle$, 15 min (12); $\circ$, control (20) and $\bullet$, 30 min (22); $\square$, control (16) and $\blacksquare$, 60 min (16); (b) 100 μM-acetylcholine: Δ , control (5) and $\blacktriangle$, 5 min (5); $\circ$, control (6) and $\bullet$, 15 min (6); $\square$, control (14) and $\blacksquare$, 30 min (15); (c) 2 mM-acetylcholine (with 2 mM-eserine): $\circ$, control (16) and $\bullet$, 5 min (15).

into phosphatidylinositol. It seemed likely, however, that this effect was a muscarinic effect elicited by the high agonist concentration. This was confirmed when it was found that the stimulation of phosphatidylinositol labelling that this agent evoked was abolished by atropine and was completely unaffected by tubocurarine (Table 6).

Discussion

Some years ago it was shown that radioactivity was lost from phosphatidylinositol when avian salt-gland was stimulated with acetylcholine (Hokin & Hokin, 1964). It was later proposed that the first event in phosphatidylinositol metabolism that was controlled by the stimulus was the removal of the phosphorylinositol group from phosphatidylinositol (Hokin, 1967). Similar observations on the loss of label from phosphatidylinositol in the phytohaemagglutinin-stimulated lymphocyte followed (Fisher & Mueller, 1968), although they were not interpreted in the same way. In both of these reports one limitation was that the radioactivity which was lost from phosphatidylinositol had been incorporated under conditions which were physiologically rather unusual; it was not clear to what extent extrapolation to the fate of lipid labelled under physiologically normal conditions would be valid.

Durell and his collaborators then also argued that removal of the phosphorylinositol group was stimulated: their conclusions were based on experiments which appeared to show that acetylcholine (and other neurotransmitters) would stimulate phosphatidylinositol breakdown in cell-free systems from cerebral cortex, albeit slightly (Durell & Garland, 1969; Durell *et al.*, 1969). Similar results were also claimed by Canessa de Scarnatti & Rodriguez de Lores Arnaiz (1972). Repetition of these experiments by using more specific assay methods has, however, failed to reveal any effects of acetylcholine in subcellular fractions, except in metabolically viable nerve-ending particles (Schacht & Agranoff, 1973; Lapetina & Michell, 1974).

On balance, therefore, it has recently seemed likely that removal of the phosphorylinositol group is indeed the controlled step in stimulated phosphatidylinositol metabolism but that this control cannot yet be achieved in cell-free systems (Michell & Lapetina, 1972; Lapetina & Michell, 1973b, 1974). Recently some additional direct evidence for cleavage of phosphatidylinositol in response to stimuli has come (a) from the stimulation by mitogenic serum factors of fibroblasts labelled with inositol (Ristow *et al.*, 1973) or doubly labelled with glycerol and phosphate (Diringer & Koch, 1973), and (b) from the effects of acetylcholine on inositol- or phosphate-labelled mouse pancreas (Hokin, 1973). Further, Hokin (1973) showed that in the pancreas as much as 40% of the phosphatidylinositol, measured chemically, could disappear in response to stimulation.

The data in the present paper establish that in the parotid gland the breakdown of phosphatidylinositol is a very early response to acetylcholine; this breakdown has been measured both radiochemically and chemically. Further, a considerable proportion of the tissue's phosphatidylinositol is involved in the

Table 3. *Simultaneous fall in phosphatidylinositol concentration and increase in incorporation of $^{32}\text{P}_i$ into phosphatidylinositol*

Unlabelled tissue fragments were incubated for 60min in radioactive medium. They were then transferred to fresh radioactive medium with or without the addition of acetylcholine ($10\mu\text{M}$) and eserine ($100\mu\text{M}$) and incubated for a further 30min. Results are quoted as mean $\pm$ S.E.M. (number of tissue samples analysed).

	Ratios of	
	^{32}P incorporation (c.p.m. in phosphatidylinositol/ c.p.m. in other phospholipids)	Phosphate content (phosphatidylinositol/ other phospholipids)
No additions	0.27 ± 0.01 (4)	0.117 ± 0.008 (7)
+ Acetylcholine and eserine	1.06 ± 0.03 (11)*	0.079 ± 0.009 (8)†
Percentage change	293% increase	32% decrease

* Significant difference from control group ($P < 0.001$).

† Significant difference from control group ($P < 0.05$).

Table 4. *Effects of cholinergic-receptor blockade on acetylcholine-stimulated phosphatidylinositol cleavage*

Parotid fragments from ^{32}P -labelled rats were incubated in unlabelled medium for 60min. Samples were then incubated for a further 30min either alone or in the presence of acetylcholine ($10\mu\text{M}$) and eserine ($100\mu\text{M}$) and, in appropriate cases, atropine ($2\mu\text{M}$) or tubocurarine ($100\mu\text{M}$). Results are given as mean $\pm$ S.E.M. (number of observations).

	Ratio: c.p.m. in phosphatidylinositol/ c.p.m. in other phospholipids	Change (%)
No additions	0.123 ± 0.006 (13)	—
Acetylcholine and eserine	0.094 ± 0.004 (15)*	-24
Acetylcholine, eserine and atropine	0.149 ± 0.007 (10)‡	+21
Acetylcholine, eserine and tubocurarine	0.081 ± 0.004 (15)†	-34

* Significant difference from control ($P < 0.001$).

† Significant difference from control ($0.001 < P < 0.01$).

‡ Significant difference from control ($0.01 < P < 0.05$).

Table 5. *Effects of methacholine on phosphatidylinositol breakdown and on $^{32}\text{P}_i$ incorporation into phosphatidylinositol*

Experimental details were exactly as for Table 3, except that methacholine ($20\mu\text{M}$) was substituted for acetylcholine and eserine. The concentration of atropine was $35\mu\text{M}$.

	Ratios of	
	^{32}P incorporation (c.p.m. in phosphatidylinositol/ c.p.m. in other phospholipids)	Phosphate content (phosphatidylinositol/ other phospholipids)
No additions	0.36 ± 0.04 (5)	0.126 ± 0.008 (5)
Methacholine	1.40 ± 0.08 (10)*	0.111 ± 0.007 (9)†
Methacholine and atropine	0.46 ± 0.02 (9)	0.131 ± 0.007 (9)

* Significant difference from control group ($P < 0.001$).

† Significant difference from control group ($0.01 < P < 0.05$).

response, as in the pancreas (Hokin, 1973). These observations therefore appear to argue against the existence, at least in these two tissues, of small metabolically independent, and possibly structurally localized, pools of phosphatidylinositol which are in some way linked to reception of stimuli but dissociated from the general metabolism of tissue

phosphatidylinositol. In a previous discussion a similar conclusion was reached on the basis of the quite different, but complementary, approaches of subcellular fractionation and radioautography of stimulated tissues (Lapetina & Michell, 1973a). However, in those earlier studies the newly labelled phosphatidylinositol, presumably a secondary pro-

Table 6. *Effects of tetramethylammonium on phosphatidylinositol breakdown and on $^{32}\text{P}_i$ incorporation into phosphatidylinositol*

Experimental details were as in Table 3, except that tetramethylammonium was substituted for acetylcholine and eserine. Other additions were atropine (50 μM) or tubocurarine (100 μM).

	Ratio of	
	^{32}P incorporation (c.p.m. in phosphatidylinositol/ c.p.m. in other phospholipids)	Phosphate content (phosphatidylinositol/ other phospholipids)
No additions	0.78 ± 0.06 (5)	0.174 ± 0.025 (5)
Tetramethylammonium (10 μM)	0.87 ± 0.06 (5)	0.163 ± 0.014 (5)
Tetramethylammonium (100 μM)	0.83 ± 0.03 (5)	0.141 ± 0.018 (5)
Tetramethylammonium (1mM)	1.78 ± 0.21 (5)*	0.108 ± 0.011 (5)†
No additions	0.44 ± 0.02 (5)	
Tetramethylammonium (1mM)	0.91 ± 0.08 (5)‡	
Tetramethylammonium (1mM) and atropine	0.47 ± 0.08 (5)	
Tetramethylammonium (1mM) and tubocurarine	1.00 ± 0.15 (5)†	

* Significant difference from control group ($P \sim 0.01$).

† Significant difference from control group ($P \sim 0.05$).

‡ Significant difference from control group ($P < 0.01$).

duct of phosphatidylinositol cleavage followed by synthesis, was assumed to be made at the endoplasmic reticulum: its rapid distribution throughout the cell was probably catalysed by cytoplasmic phosphatidylinositol exchange protein(s).

Although the present results indicate that much of the phosphatidylinositol of cells can be cleaved in response to stimulation, this does nothing to clarify the intracellular site at which the cleavage occurs. The data are equally compatible either with a widespread effect throughout the cell or with a response at a localized site, to which the phosphatidylinositol transfer protein brings a continuous supply of substrate. Several arguments have previously inclined us to the latter view, with the plasma membrane as the most probable location (Lapetina & Michell, 1973a). Clearly there is a need for short-term experiments which attempt to define the subcellular distribution of phosphatidylinositol loss in cells receiving intense stimulation.

If, as suggested above, the phosphatidylinositol involved in response to a stimulus belongs to a large pool which includes much of the phosphatidylinositol of the responding cell, then why have changes in tissue phosphatidylinositol concentration not been observed by previous workers (Karnovsky & Wallach, 1961; Larrabee & Leicht, 1965; Gaut *et al.*, 1966; Gaut & Huggins, 1966; Pumphrey, 1969; Trifaró, 1969)? Three factors seem likely to contribute to this situation. First, the abilities of different tissues to resynthesize lost phosphatidylinositol may vary. Such variation might be a consequence of differences in the availability of appropriate cofactors and precursors such as cytidine and inositol (see the Results section) and possibly also of the differing complements of

biosynthetic enzymes in various tissues. Secondly, the intensity of stimulation modulates the rate of phosphatidylinositol breakdown. Thus it seems likely that under the influence of stimulation of normal physiological intensity the important result would be an increase in the rate of turnover of the phosphorylinositol part of the molecule, rather than a marked change in the lipid composition of cellular membranes. Thirdly, if the responding cell population only contains a small proportion of the phosphatidylinositol of the whole tissue then even the loss of a large fraction of this under intense stimulation may lead to an insignificant change in the overall tissue phosphatidylinositol concentration. For example, the radioautographic studies of sympathetic ganglia only reveal enhanced labelling in neuronal perikarya, but these form a minor proportion of the tissue mass. A similar explanation may apply to the small pool of responsive phosphatidylinositol in the avian salt gland (Hokin & Hokin, 1964; Hokin, 1967).

In the experiments of Hokin (1973) it was shown that the decrease in pancreatic phosphatidylinositol concentration produced by acetylcholine was balanced by the accumulation within the tissue of an equivalent quantity of newly synthesized phosphatidic acid; this was presumably synthesized by the diacylglycerol kinase route, by using the 1,2-diacylglycerol liberated from phosphatidylinositol as substrate. The fate of the liberated diacylglycerol has not been determined in our experiments, since our studies of phosphatidylinositol labelling demonstrate that in the Krebs-Ringer bicarbonate buffer that we were using the incorporation of P_i is markedly stimulated by the addition of inositol or cytidine. This suggests that in this medium the form of the lipid undergoing recycling to phosphatidyl-

inositol is, at any particular time, going to be determined by the availability of substrates and cofactors to the tissue fragments, rather than to a physiologically meaningful rate-limiting step.

The experiments with cholinergic agonists and blockers show quite clearly that the cholinergic receptors involved in these phenomena are muscarinic; the muscarinically acting agents methacholine and atropine showed potent effects, whereas the nicotinically acting tetramethylammonium and tubocurarine were without appreciable effects at low concentrations. Previous studies, though less detailed, have generally led to the same conclusion (see Schacht & Agranoff, 1972). This is one reason for doubting the model of De Robertis (1971), which envisages phosphatidylinositol as a component of the nicotinic cholinergic receptor. There is one report which includes an observation of blockade by tubocurarine of electrical stimulation of phosphatidylinositol turnover in superior cervical sympathetic ganglia (Larrabee & Leicht, 1965). We have not, however, been able to observe this effect of tubocurarine in ganglia incubated with acetylcholine (R. H. Michell & W. E. Brown, unpublished work).

Possible functions for enhanced turnover of the phosphorylinositol group of phosphatidylinositol include intracellular production of *myo*-inositol 1,2-cyclic phosphate (Michell & Lapetina, 1972) and changes in the lipid patterns of cell membranes (see Hawthorne, 1973; Lapetina & Michell, 1973a, for reviews). The suggestion that the production of the cyclic ester might be of physiological significance has been criticized by Freinkel & Dawson (1973) who failed to find either a function for or the intracellular existence of either this compound or *myo*-inositol 1-phosphate. However, both the data presented here and those of Hokin (1973) indicate that some form of inositol phosphate must be produced in stimulated cells, even if only transiently. Further, the existence in liver of inositol 1-phosphate, or of a molecule that is converted into inositol 1-phosphate under acid conditions, has been known for many years (Hübscher & Hawthorne, 1957; Galliard & Hawthorne, 1963) and we have preliminary evidence for the existence of inositol 1:2-cyclic phosphate in cerebral cortex *in vivo* (Michell & Lapetina, 1973). Further work is clearly required to resolve this key question.

We are grateful to the Medical Research Council for generous financial support and to Dr. D. Allan, Dr. R. Coleman and Dr. J. B. Finean for their comments.

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