Enhanced Phosphatidylinositol Labelling in Rat Parotid Fragments Exposed to α -Adrenergic Stimulation

By ROBERT H. MICHELL and LYNNE M. JONES
*Department of Biochemistry, University of Birmingham, P.O. Box 363,
Birmingham B15 2TT, U.K.*

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1. Adrenergic agonists provoke a marked increase in labelling of phosphatidylinositol in fragments of rat parotid gland. 2. Adrenaline and phenylephrine (an adrenergic α -agonist) are effective stimulants, but isoprenaline (an adrenergic β -agonist) is relatively ineffective. 3. The response evoked by phenylephrine or adrenaline is prevented by prior incubation of the tissue with phenoxybenzamine (an α -receptor blocking agent), but not by prior incubation with pindolol (a β -receptor blocking agent). 4. Adrenergic stimulation of phosphatidylinositol metabolism in parotid gland is therefore mediated through α -receptors, in common with the adrenaline-induced K^+ efflux. It is not linked to enzyme secretion, which is triggered by stimulation of β -receptors. 5. It is suggested that the stimulation of phospholipid metabolism that occurs in several other tissues in the presence of adrenaline or noradrenaline may also involve α -receptors.

Many tissues show an increased incorporation of $^{32}P_i$ into phosphatidylinositol when stimulated with hormones or neurotransmitters (L. E. Hokin, 1968; Michell & Lapetina, 1972; Lapetina & Michell, 1973a). In tissues that secrete proteins a link has been sought between the enhancement of phosphatidylinositol labelling and the secretory process (see L. E. Hokin, 1968), but a direct link between these processes has recently seemed less likely (L. E. Hokin, 1966, 1971; Sandhu & Hokin, 1967; M. R. Hokin, 1968, 1970; Lapetina & Michell, 1973a; Gerber *et al.*, 1973).

The parotid gland is an exocrine secretory organ which responds to a wide range of agents, the response including secretion of enzymes (Schramm, 1967; Babad *et al.*, 1967; Batzri & Selinger, 1973; Batzri *et al.*, 1973), efflux of K^+ ions (Batzri *et al.*, 1973), and changes in phosphatidylinositol metabolism (Hokin & Sherwin, 1957; Eggman & Hokin, 1960; Sandhu & Hokin, 1967). In the rat parotid gland the effects of adrenaline are mediated through two different classes of receptor, with β -receptors controlling protein secretion and α -receptors controlling K^+ efflux (Batzri & Selinger, 1973; Batzri *et al.*, 1973). The purpose of the experiments described here was to determine which type of receptor is responsible for the control of phosphatidylinositol turnover.

Materials and Methods

Materials

Inosine, adenosine and 2-hydroxybutyrate were obtained from Sigma (London) Chemical Co.,

Kingston-upon-Thames, Surrey KT2 7BH, U.K. Adrenaline was from BDH Ltd., Poole, Dorset, U.K., phenoxybenzamine (Dibenyline) from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts., U.K., pindolol (LB 46) from Sandoz Products Ltd., London W1X 0AL, U.K., phenylephrine from Boots Pure Drug Co. Ltd., Nottingham, U.K., and isoprenaline from Vestric Ltd., Brierley Hill, Staffs., U.K. The structures of these reagents are given in Goodman & Gilman (1970) and in Singh & Vaughn-Williams (1971). Other materials were obtained from sources previously described (Lapetina & Hawthorne, 1971; Lapetina & Michell, 1972).

Methods

The medium used was a modified Krebs–Ringer bicarbonate medium containing 14 mM- K^+ and 5 mM-2-hydroxybutyrate (Babad *et al.*, 1967), 10 mM-inosine and 0.5 mM-adenine (Batzri & Selinger, 1973), and $^{32}P_i$ (5 or 10 $\mu Ci/ml$). The medium was gassed with $O_2 + CO_2$ (95:5) before the experiment and incubation mixtures were gassed at intervals of 30–60 min during the experiments. The incubations were at 37°C in 50 ml conical flasks in a shaking incubator.

The usual experimental design was (a) to collect parotid glands and to prepare fragments of these, (b) to incubate them in ^{32}P -labelled medium for about 1 h to label intracellular precursor pools, and (c) to measure the effects of added agents on the phospholipid labelling that occurred in the fragments over the subsequent 30 min. When receptor-blocking agents were tested these were added 20 min before the start of the final 30 min period.

Four to six Wistar rats of either sex (about 250g body wt.) were used for each experiment. They were usually starved overnight (Batzri & Selinger, 1973); this had little or no effect on the observed ^{32}P incorporations. The parotid glands were removed, chopped into fragments (1mm^2) with a McIlwain tissue chopper (Mickle Instruments Ltd.) and the fragments from each pair of glands incubated in 10ml of radioactive medium. This procedure took about 10min for each rat. After all glands had been incubating for at least 50–60min the fragments were recovered by rapid filtration (Whatman no. 41 paper), the fragments from all glands pooled and the mixture was divided into an appropriate number of approximately equal samples. (In experiments with antagonists the glands were first collected in non-radioactive medium, then pooled and divided into two portions. These were then incubated with 10ml of radioactive medium, the antagonist being added to one portion after 40min. After 60min they were collected, divided and the method was followed as described below.)

The portions, each equivalent to about one-fifth of one pair of glands, were put into flasks containing 4ml of fresh radioactive medium and were incubated in the presence or absence of agonists and antagonists (added at appropriate concentrations in a volume of 0.04ml). After 30min the fragments were recovered by filtration, washed once with non-radioactive medium and transferred into homogenizer flasks containing 1.5ml of 2M-KCl and 5.6ml of chloroform-methanol (1:2, v/v). They were homogenized with an MSE top-drive homogenizer, the homogenates transferred to tubes and the homogenization vessels rinsed with 1.9ml of 2M-KCl and 1.9ml of chloroform. The contents of the tubes were mixed and centrifuged, the lower phases collected and the upper phase was washed as described previously (Michell *et al.*, 1970; Lapetina & Michell, 1972). The lipid extracts were dried by using a Buchler Rotary Evaporator (Arnold R. Horwell Ltd.) and phosphatidylinositol was separated and analysed for phosphate and radioactivity (Lapetina & Michell, 1972, 1973b). The major faster-running spot on the papers, of which the major constituent is phosphatidylcholine, was also analysed.

Results

Stimulation of phosphatidylinositol labelling by adrenaline

The marked effect of adrenaline on phosphatidylinositol labelling is shown in Fig. 1. Clearly the addition of adrenaline to parotid fragments after 60min of incubation caused a marked increase in the rate of incorporation of ^{32}P into this lipid during the subsequent 30min. This experimental design was chosen to allow considerable labelling of the precursor pools in

Fig. 1. *Effect of adrenaline on labelling of phospholipids*

Tissue fragments were incubated in radioactive medium for 60min or for 90min. Adrenaline ($30\mu\text{M}$) was added at 60min to the samples designated by solid symbols (●, ■). Phosphatidylinositol is represented by (○, ●) and (□, ■) represents the major fast-running phospholipid area (chiefly phosphatidylcholine). Data are the means of triplicate incubations.

the tissue before addition of the stimulus, and thus to minimize effects caused by any changes in the entry of label that might be produced by stimulation.

Further evidence to suggest that the enhanced phosphatidylinositol labelling was not a consequence of enhanced labelling of precursor pools is provided by the large increase in phosphatidylinositol labelling relative to that of other lipids (Fig. 1). This comparison is striking, even though the 'phosphatidylcholine' samples will have included phosphatidic acid, whose labelling will almost certainly have been stimulated to some degree (Eggman & Hokin, 1960).

The effect of adrenaline was dose-dependent (Fig. 2), with half-maximum stimulation at approx. $30\mu\text{M}$. This concentration is very close to that which produces half-maximum K^+ efflux and considerably higher than that required to elicit half-maximum amylase secretion [data of Batzri *et al.* (1973), plotted in Fig. 2].

Effects of phenylephrine and isoprenaline

Phenylephrine specifically stimulates adrenergic α -receptors and isoprenaline stimulates β -receptors (Goodman & Gilman, 1970). Phenylephrine was very effective in provoking phosphatidylinositol turnover,

but isoprenaline was ineffective at concentrations up to 300 μM (Table 1).

Effects of α-receptor blockade

When parotid fragments were incubated with phenoxybenzamine, an agent which blocks adrenergic α-receptors (Goodman & Gilman, 1970), before addition of phenylephrine they no longer showed any change in phosphatidylinositol labelling in response to stimulation (Figs. 3a and 3b). There was a slight increase in labelling owing to the presence of phenoxybenzamine, but as this was not matched by an increase in the ratio of the labelling of phosphatidylinositol

to that of ‘phosphatidylcholine’ it probably reflected a slight increase in tissue permeability to phosphate.

Effects of β-receptor blockade

Pindolol (LB46) is a highly potent β-receptor blocker with little of the ‘local anaesthetic’ or ‘membrane-active’ properties associated with many β-blockers (Singh & Vaughn-Williams, 1971). The response of parotid phospholipid metabolism to adrenaline or phenylephrine was not abolished by the presence of this compound either at 10 μM (Figs. 3c and 3d) or at 100 μM.

Fig. 2. Effect of adrenaline on phosphatidylinositol labelling

Tissue fragments were incubated in radioactive medium for 60 min (designated zero time) or for 90 min (adrenaline being added for the final 30 min). The symbols (●) and solid curve show the increase in phosphatidylinositol labelling during the final 30 min of incubation. The other curves, which are drawn from data in Fig. 3 of Batzri *et al.* (1973), show the effect of adrenaline on K⁺ efflux (----) and on amylase secretion (····). Incubations were in duplicate.

Discussion

Receptors that mediate enhancement of phosphatidylinositol turnover

Several pieces of evidence presented above identify the adrenergic receptors that mediate the stimulation of phosphatidylinositol metabolism as α-receptors: (a) they are sensitive to phenylephrine and relatively insensitive to isoprenaline; (b) they are blocked by preincubation with phenoxybenzamine but not with pindolol; and (c) enhancement of phosphatidylinositol labelling occurs at adrenaline concentrations similar to those needed to provoke K⁺ efflux, rather than at the lower concentrations which elicit protein secretion (Batzri *et al.*, 1973). The mechanism by which stimulation of α-receptors in the parotid provokes K⁺ efflux and phosphatidylinositol turnover is not known. The hypothesis (Sutherland, 1965; Robison *et al.*, 1971, pp. 145–231) that α-receptors act through a depression of the overall tissue concentration of cyclic AMP is clearly not tenable in this tissue, because K⁺ efflux occurs in response to stimulation of α-receptors even when the tissue cyclic AMP concentration is high (Batzri *et al.*, 1973).

Table 1. Effects of adrenergic agonists on phosphatidylinositol labelling

Tissue fragments were incubated in radioactive medium for 60 or 90 min. Where appropriate, adrenergic agents were present (at the concentration specified) during the final 30 min of incubation. Incubations were in duplicate.

Agonist	Concentration (μM)	Incubation period (min)	Phosphatidylinositol labelling (c.p.m./nmol)
None	—	60	31
None	—	90	36
Adrenaline	3	90	47
	30	90	228
	300	90	123
Phenylephrine	3	90	68
	30	90	135
	300	90	120
Isoprenaline	3	90	47
	30	90	38
	300	90	51

Fig. 3. Effects of α - and β -receptor blockade on phenylephrine-stimulated phosphatidylinositol labelling

Tissue fragments were incubated in radioactive medium for 60 min (left-hand bar in each panel) or for 90 min. Phenylephrine (30 μM) was present for the last 30 min in the samples on the right of each panel. Phenoxybenzamine (10 μM) or pindolol (10 μM) were present in all incubations in, respectively, panels (b) and (d) during the last 50 min of incubation. Incubations were in triplicate and the data in panels (a) and (b) and in panels (c) and (d) came from separate experiments. The ratio is the total radioactivity of phosphatidylinositol divided by the total radioactivity of the major fast-running phospholipid area (chiefly phosphatidylcholine).

Relationship between phosphatidylinositol metabolism and protein secretion

Protein secretion by the rat parotid is mediated through stimulation of adrenergic β -receptors that produce their action through an increase in the tissue cyclic AMP concentration (Batzri & Selinger, 1973). Such an increase does not provoke enhanced phosphatidylinositol turnover either in parotid gland (Sandhu & Hokin, 1967) or elsewhere (Baudhuin *et al.*, 1971; Baudhuin & Cantraine, 1972; De Torrentegui & Berthet, 1966; Eichberg *et al.*, 1973a,b; Kerkof & Tata, 1969; Schnell-Frederick & Dumont, 1970; Robison *et al.*, 1971, pp. 353–355).

Thus the demonstration that stimulated phosphatidylinositol labelling is mediated through a receptor different from that controlling protein secretion adds a further item to the substantial body of information which indicates that there is not a direct connection between stimulation of phosphatidylinositol labelling and secretion of macromolecules either in the parotid gland or elsewhere (M. R. Hokin, 1968, 1970; Trifaró, 1969a,b; Michell & Lapetina, 1972; Lapetina & Michell, 1973a; Gerber *et al.*, 1973).

Effects of adrenergic agonists and phospholipid metabolism in other tissues

Adrenergic agents modify phospholipid metabolism in a variety of tissues. Usually phospholipid labelling from $^{32}\text{P}_i$ is stimulated [parotid gland (Hokin & Sherwin, 1957; Sandhu & Hokin, 1967; the present paper), submaxillary gland (Hokin & Sherwin, 1957; Gaut *et al.*, 1966; Burford & Huggins, 1963), heart (Gaut & Huggins, 1966), liver (De Torrentegui & Berthet, 1966), brain (M. R. Hokin, 1969; Sneddon & Keen, 1970), pineal gland (Eichberg *et al.*, 1973a,b) and adipose tissue (Stein & Hales, 1972)], and in the one case in which inhibition was reported [thyroid (Altmann *et al.*, 1966)] the concentration used was very high; a similar concentration applied to parotid is inhibitory (L. Jones & R. H. Michell, unpublished work). Usually the most marked effects were on phosphatidylinositol and phosphatidic acid, but in two studies the effects appeared to be somewhat less specific (De Torrentegui & Berthet, 1966; Stein & Hales, 1972). In most of these studies prevention by α -receptor blockade of the adrenaline-induced changes in lipid metabolism was demonstrated; pineal gland may be exceptional in this respect (Eichberg *et al.*, 1973a,b). It thus seems likely that stimulation of phosphatidylinositol metabolism is quite a widespread response of tissues to α -adrenergic stimulation.

Note added in proof (received 8 October 1973)

In a recent brief report Oron *et al.* (1973) have described experiments basically similar to those reported here, but in which different α - and β -blocking agents were used; their conclusions were similar to ours.

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