Muscarinic Cholinergic Stimulation of Phosphatidylinositol Turnover in the Longitudinal Smooth Muscle of Guinea-Pig Ileum

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1. The metabolism of phosphatidylinositol and phosphatidate was investigated in fragments of longitudinal smooth muscle from guinea-pig ileum incubated with cholinergic and anticholinergic drugs. 2. Incorporation of P_i into these lipids was enhanced by acetylcholine and carbamoylcholine. 3. The receptor responsible for triggering this response was of the muscarinic type, since (a) the response was also produced by the muscarinic agonists acetyl- β -methylcholine, carbamoyl- β -methylcholine and pilocarpine, and (b) the response was prevented by atropine and propylbenzilylcholine mustard, but not by tubocurarine. 4. Increased phosphatidylinositol labelling was clearly observed within 5 min in tissue treated with a high concentration of carbamoylcholine. 5. Half-maximal stimulation of phosphatidylinositol labelling occurred at approx. $10 \mu M$ -carbamoylcholine. 6. Incubation of muscle fragments with carbamoylcholine provoked a decrease in phosphatidylinositol concentration, as would be expected if phosphatidylinositol breakdown is the reaction controlled by agonists. 7. This information all appears consistent with the proposal that phosphatidylinositol breakdown may be a reaction intrinsic to the mechanisms of muscarinic cholinergic receptor systems.

A large variety of tissues show increased incorporation of $^{32}P_i$ into phosphatidylinositol and into phosphatidate, its biosynthetic precursor, when they are exposed to certain hormones and neurotransmitters. Many of these tissues show this response when stimulated by either muscarinic cholinergic or α -adrenergic agonists, and it has been suggested that the increased metabolism of phosphatidylinositol may play a central role in the mechanisms of these receptors (Michell, 1975; Michell *et al.*, 1976).

If the phosphatidylinositol response is involved in the mechanisms of the receptor systems then an essential prediction is that all tissues which possess the appropriate receptor systems must show the response. The longitudinal smooth muscle of guinea-pig ileum was chosen as the initial system in which to test this prediction, because pharmacological studies have shown that it contains receptors for a variety of physiological agonists. In particular, it is very rich in muscarinic cholinergic receptors, and for many years it has been one of the standard systems for the pharmacological and electrophysiological characterization of these receptors (see, for example, Triggle, 1971; Bülbring *et al.*, 1970). Recently a phosphatidylinositol response to α -adrenergic stimuli has been observed in two other smooth muscles, namely rat vas deferens and rabbit iris (Canessa de Scarnatti & Lapetina, 1974; Abdel-Latif, 1974, 1975), and a response to muscarinic cholinergic stimulation

was also found in the iris muscle (Abdel-Latif, 1974, 1975).

The purpose of our experiments was to determine whether the longitudinal smooth muscle of guinea-pig ileum exhibited a phosphatidylinositol response to cholinergic stimuli. Having found that it showed this response, the receptor which triggered the response was then investigated pharmacologically to determine whether it was of the muscarinic type. Some of these experiments were reported briefly (Michell *et al.*, 1976).

Materials and Methods

Materials

Cholinergic agonists were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., as were atropine sulphate and eserine sulphate. Tubocurarine was from Burroughs-Wellcome and Co., Temple Hill, Dartford, Kent, U.K. Propylbenzilylcholine mustard was a generous gift of Dr. J. M. Young of the Department of Pharmacology, University of Cambridge.

Methods

The basic experimental design was to compare the labelling of fragments of the longitudinal smooth muscle from guinea-pig ileum incubated in ^{32}P -labelled Krebs–Ringer bicarbonate medium (Krebs

& Henseleit, 1932) in the presence and in the absence of cholinergic drugs; the addition of antagonists usually preceded that of agonists and unless otherwise specified the chosen agonist was present during the final 30 min period of a 60 min incubation.

The ileum was removed from two or three female guinea pigs (weight approx. 400g), and the longitudinal smooth muscle obtained as described by Rang (1964). The muscle was cut into small pieces, and the fragments were distributed into flasks containing 3 ml of unlabelled Krebs-Ringer bicarbonate medium (Krebs & Henseleit, 1932) containing 11 mM-glucose. Then 1 ml of the same medium, containing $^{32}\text{P}_i$ (about 40 $\mu\text{Ci/ml}$), was added and the flasks were gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and incubated at 37°C in a shaking incubator. In most experiments antagonists were added at the same time as the $^{32}\text{P}_i$ and agonists after 30 min. At the end of the incubation period, which was usually 60 min, the fragments were quickly recovered by filtration, washed with ice-cold buffer and transferred into homogenization vessels containing 1.5 ml of 2 M-KCl and 5.6 ml of chloroform/methanol (1:2, v/v). Extraction of the tissue lipids was as described by Michell & Jones (1974). The lipid extracts were analysed by descending chromatography on formaldehyde-treated papers in (a) butan-1-ol/acetic acid/water (4:1:5, by vol.) (Michell & Jones, 1974) or (b) butan-1-ol/formic acid/water (4:1:5, by vol.) (Allan *et al.*, 1975). In the former solvent phosphatidylinositol is well resolved from other lipids and separates as a compact spot, whereas phosphatidate is a component of the mixed spot which contains most of the tissue lipids (see Allan & Michell, 1975). In solvent (b), on the other hand, the spots are somewhat less compact, but phosphatidate moves near the solvent front together with the phosphorus-free non-polar lipids: this solvent could therefore be used for determination of the behaviour of phosphatidate, but

solvent (a) was preferred when only phosphatidylinositol was to be analysed. The separated lipid spots were detected, digested, analysed for phosphorus, and their radioactivity was determined by methods described previously (Lapetina & Michell, 1972, 1973; Michell & Jones, 1974). The results were calculated as the specific radioactivities of phosphatidylinositol and, in some cases, of phosphatidate.

Results

Stimulation of phosphatidylinositol labelling by carbamoylcholine and other cholinergic agonists

Carbamoylcholine is a cholinomimetic drug which stimulates both muscarinic and nicotinic receptors and which is not susceptible to hydrolysis by cholinesterases. At a concentration of 100 μM it caused marked stimulation of the labelling of both phosphatidate and phosphatidylinositol in smooth-muscle fragments (Table 1). The major lipid spot, which contained most of the other phospholipids of the tissue, showed very little labelling during a 1 h incubation, whether carbamoylcholine was present or not (results not shown).

The effect of carbamoylcholine was concentration-dependent, with half-maximum response at about 10 μM (Fig. 1); this is a concentration much higher than is needed to elicit half-maximum contraction of this tissue.

The response was readily detected within 5 min at a high carbamoylcholine concentration (Fig. 2a), and the stimulated incorporation of ^{32}P into phosphatidylinositol was approximately proportional to the period of incubation for about 50 min, after which it began to decrease (Fig. 2b).

Other cholinergic agonists that elicited the response were 100 μM -acetylcholine (in the presence of 10 μM - eserine), 100 μM -acetyl- β -methylcholine (methacholine), 100 μM -carbamoyl- β -methylcholine (bethane-

Table 1. Increased labelling of phosphatidylinositol and phosphatidate with ^{32}P in response to carbamoylcholine

All tissue fragments were incubated with $^{32}\text{P}_i$ for 30 min. Some incubations were then terminated, and the remaining tissue was incubated with or without 100 μM -carbamoylcholine for a further 30 min. Values are means $\pm$ S.E.M. of results from triplicate incubations in one of approximately ten similar experiments.

	Specific radioactivities (d.p.m./nmol)	Increase in ^{32}P labelling during second 30 min period	
		(d.p.m./nmol)	(% of control)
Phosphatidate			
Incubated 30 min	49 $\pm$ 2		—
Incubated 60 min	67 $\pm$ 5	18	(100)
Incubated 60 min with carbamoylcholine	121 $\pm$ 9*	72	400
Phosphatidylinositol			
Incubated 30 min	39 $\pm$ 7		—
Incubated 60 min	76 $\pm$ 9	37	(100)
Incubated 60 min with carbamoylcholine	149 $\pm$ 1*	110	297

* Significantly different from equivalent incubation without carbamoylcholine ($P < 0.01$).

Fig. 1. Effect of carbamoylcholine concentration on phosphatidylinositol labelling

Tissue fragments were incubated in ^{32}P -labelled medium for 30 min in the absence of carbamoylcholine and for a further 30 min in the presence of the stated concentration of the drug. Values given are mean specific radioactivities of phosphatidylinositol in duplicate or triplicate incubations at the end of this period; they are taken from one of three similar experiments.

chol) and 1 mM-pilocarpine (Table 2). Of these compounds, acetylcholine and carbamoylcholine are fairly non-specific cholinergic agonists, which stimulate both muscarinic and nicotinic receptors, whereas acetyl- β -methylcholine, carbamoyl- β -methylcholine and pilocarpine only affect muscarinic receptors.

Effects of cholinergic receptor blockade on carbamoylcholine-stimulated phosphatidylinositol labelling

Tubocurarine, which is specific blocker of nicotinic receptors, caused a decrease in the labelling of phosphatidylinositol in both control and carbamoylcholine-treated tissue. It did not, however, cause any appreciable change in the response to carbamoylcholine (Table 3). On the other hand, the muscarinic antagonist atropine completely prevented the stimulation of phosphatidylinositol labelling by carbamoylcholine (Table 3). These are the same results as have been reported in the past for a variety of other tissues (see Michell, 1975).

Further information on the nature of the receptor involved in triggering the response was sought by using propylbenzilylcholine mustard, a highly specific muscarinic-blocking agent (Burgen *et al.*, 1974); pretreatment with this drug completely prevented the

Fig. 2. Time-course of phosphatidylinositol labelling in response to carbamoylcholine

●, Control incubations; ▲, incubations in the presence of carbamoylcholine. Experimental details for the experiment of (a) were as for Table 3, except that lipids were separated by chromatography in solvent (a) and a higher concentration of carbamoylcholine (1 mM) was used. ----, Approximately linear incorporation of $^{32}\text{P}_i$ that occurs during incubation of tissue fragments in the absence of carbamoylcholine (see Table 1 and Fig. 2b); —, incorporation observed when carbamoylcholine was added after 30 min of incubation. The results presented are mean values of phosphatidylinositol specific radioactivity in either four or five identical incubations. In (b) an experiment is shown in which the usual concentration of carbamoylcholine (100 μM) was used, and $^{32}\text{P}_i$ and carbamoylcholine were present throughout the incubations. Each point is the result from a single incubation, and the results presented are from one of three similar experiments.

response of phosphatidylinositol labelling to carbamoylcholine (Table 4).

Stimulation of phosphatidylinositol breakdown by carbamoylcholine

In a number of experiments the phosphatidylinositol concentrations of control and carbamoylcholine-treated tissue were compared, and there appeared to be a slight, but often statistically insignificant, decrease caused by stimulation. When the results from several experiments, in which stimulation with 100 μ M-carbamoylcholine was for 30–120 min (30 min, five experiments; 60 min, two experiments; 120 min, two experiments), were combined it became clear that there was a small, but highly significant, cholinergically stimulated breakdown of phosphatidylinositol. The results, expressed

as a ratio of the concentration of phosphatidylinositol to that of the spot containing most of the tissue lipids, were 0.076 ± 0.0025 (36) for the control tissue samples and 0.066 ± 0.0025 (42) for the tissue incubated with carbamoylcholine (means $\pm$ S.E.M., with the number of tissue samples analysed given in parentheses). These values were significantly different ($P < 0.01$).

Discussion

Cholinergic agonists stimulate phosphatidylinositol metabolism in tissues of many types; for example, pancreas, thyroid, parotid gland, sweat glands, cerebral cortex and iris smooth muscle (see Michell, 1975; Michell *et al.*, 1976). In the present paper we report the same effect in the longitudinal smooth muscle of guinea-pig ileum.

The contractile response of various types of smooth-muscle tissues in response to cholinergic stimulation is well known. In the guinea-pig ileum, as in other smooth muscles, this effect is mediated through activation of muscarinic receptors. Contraction is also the physiological response of this tissue to a range of other agonists, including histamine, 5-hydroxytryptamine, angiotensin II, prostaglandin $F_{2\alpha}$ and noradrenaline (in the presence of β -receptor blockade). At least three of these other agonists also trigger increased incorporation of $^{32}\text{P}_i$ into phosphatidylinositol very soon after their presentation to the tissue (Jafferji & Michell, 1976). These observations appear to be consistent with our recent suggestion (Michell, 1975; Michell *et al.*, 1976) that phosphatidylinositol breakdown, the first reaction in the cycle responsible for increased phosphatidylinositol labelling, may be an early and essential step in the receptor-activation pathway, which leads ultimately to contraction. We have confirmed that in ileum smooth muscle, as in pancreas (Hokin-

Table 2. *Stimulation of phosphatidylinositol labelling by muscarinic cholinergic agonists*

Experimental details are as for Table 1, except that all incubations were for 60 min: drugs were present during the last 30 min of incubation. Values given are of phosphatidylinositol specific radioactivity as a percentage of the control value. From two to five experiments (numbers in parentheses) were performed with each drug.

	Phosphatidylinositol specific radioactivity (% of control value)
No drugs added	(100)
Carbamoylcholine (100 μ M)	190 (5)
Carbamoyl- β -methylcholine (100 μ M)	170 (2)
Acetylcholine (100 μ M) + eserine (10 μ M)	190 (3)
Acetyl- β -methylcholine (100 μ M)	140 (3)
Pilocarpine (1 μ M)	220 (2)

Table 3. *Effect of atropine and tubocurarine on the increased labelling of phosphatidylinositol in response to carbamoylcholine*

Tissue fragments were incubated with $^{32}\text{P}_i$ for 10 min either with or without 100 μ M-carbamoylcholine, 1.7 μ M-atropine or 1.44 μ M-tubocurarine. Values are means $\pm$ S.E.M. (number of incubations in parentheses) and are taken from one of three similar experiments.

	Specific radioactivity of phosphatidylinositol (d.p.m./nmol)	^{32}P incorporation as percentage of appropriate control value without carbamoylcholine
No additions	25 ± 3 (2)	(100)
Carbamoylcholine	50 ± 3 (3)*	200
Atropine	27 ± 3 (4)	(100)
Carbamoylcholine and atropine	25 ± 2 (5)	93
Tubocurarine	17 ± 2 (5)	(100)
Carbamoylcholine and tubocurarine	36 ± 2 (3)†	213

* Significantly different from equivalent incubation without carbamoylcholine ($0.01 < P < 0.05$).

† Significantly different from equivalent incubation without carbamoylcholine ($P < 0.01$).

Table 4. *Prevention of cholinergically stimulated phosphatidylinositol turnover by propylbenzylcholine mustard*

Tissue fragments were incubated for 30 min with $^{32}\text{P}_i$, either with or without $12.5\ \mu\text{M}$ -propylbenzylcholine mustard. Carbamoylcholine ($100\ \mu\text{M}$) was present during a further 30 min incubation. Values are means $\pm$ S.E.M. of triplicate incubations, and are taken from one of five similar experiments.

	Phosphatidylinositol specific radioactivity (d.p.m./nmol)
No additions	230 ± 3
$100\ \mu\text{M}$ -Carbamoylcholine	$420 \pm 10^*$
$12.5\ \mu\text{M}$ -Propylbenzylcholine mustard	220 ± 12
$100\ \mu\text{M}$ -Carbamoylcholine and $12.5\ \mu\text{M}$ -propylbenzylcholine mustard	220 ± 13

* Significantly different from equivalent incubation without carbamoylcholine ($P < 0.001$).

Neaverson, 1974) and parotid gland (Jones & Michell, 1974, 1975), the reaction controlled by cholinergic stimulation is indeed phosphatidylinositol breakdown.

The pharmacological studies with atropine and propylbenzylcholine mustard confirmed that the response that we were studying was mediated through muscarinic cholinergic receptors; the benzylcholine mustards are among the most potent and specific blocking agents for receptors of this type (Gill & Rang, 1966; Burgen *et al.*, 1974), but this seems to be the first time that they have been used for pharmacological studies of a phosphatidylinositol-turnover response.

The dose-response curve of the phosphatidylinositol response to carbamoylcholine is close to the receptor-occupation curve for this agonist and tissue (Furchgott & Burtsztn, 1967), and is displaced to much higher concentrations than are required to elicit a contractile response. The slight flattening of the curve, when compared with the receptor-occupation curve and with the dose-response curves for membrane conductance and Rb^+ or K^+ efflux from the same tissue (Bolton, 1973; Burgen & Spero, 1968), may be due to the use of phosphatidylinositol labelling, which is at best a semi-quantitative assay (Michell, 1975; Michell *et al.*, 1976), for determination of the dose-response curve. The general similarity between the receptor-occupation curve for carbamoylcholine and the dose-response curve for the phosphatidylinositol response is consistent with, but does not prove, the suggested role of phosphatidylinositol breakdown in receptor mechanisms (Michell *et al.*, 1976).

Obviously the present studies are only the first stage in testing whether phosphatidylinositol break-

down is integral to the muscarinic cholinergic receptors of ileum smooth muscle. They are, however, encouraging in that the predicted association between muscarinic receptors and the phosphatidylinositol-turnover response (Michell, 1975) has been found in a tissue that has been very well studied pharmacologically, but whose phospholipid metabolism has not previously been examined from this point of view. This should considerably facilitate future attempts to define the function of cholinergically stimulated phosphatidylinositol metabolism.

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