Cholinergically Stimulated Phosphatidylinositol Breakdown in Parotid-Gland Fragments is Independent of the Ionic Environment

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Carbamoylcholine stimulated phosphatidylinositol breakdown in rat parotid-gland fragments incubated in either a Krebs–Ringer medium or an ion-depleted iso-osmotic sucrose medium. This suggests that phosphatidylinositol breakdown is not initiated by a change in the intracellular concentration of Na^+ or of K^+ , and previous experiments have already indicated that it is independent of cyclic nucleotides and Ca^{2+} . Thus it seems that this reaction may be initiated by a direct interaction at the plasma membrane between the activated muscarinic cholinergic receptor system and the enzyme that catalyses phosphatidylinositol breakdown.

Muscarinic cholinergic stimulation provokes phosphatidylinositol breakdown in a variety of tissues, among them the rat parotid gland (Michell *et al.*, 1976). In principle, this effect might be mediated through a change in intracellular concentration of a second messenger such as cyclic AMP or cyclic GMP, through a change in the intracellular ionic environment, or through direct control of an enzyme by the activated receptor at the plasma membrane. Previous studies have already provided evidence that argues strongly against initiation of this particular response by either cyclic nucleotides or Ca^{2+} (Trifaró, 1969; Michell, 1975; Jones & Michell, 1975; Oron *et al.*, 1975; Michell *et al.*, 1976), but there has been little evidence that might discriminate between direct coupling to the activated receptor and effects of ions other than Ca^{2+} . The only previous studies that might throw a little light on this point appear to be those by Schneider (1974) on thyrotropin-stimulated thyroid gland and by Ristow *et al.* (1975) on fibroblasts exposed to mitogenic serum factors: these are two of the other situations in which activation of cell-surface receptors leads to phosphatidylinositol turnover, probably by a mechanism similar to that involved in the muscarinic cholinergic response (Michell, 1975). In both cases the response was relatively insensitive to removal of Na^+ and/or K^+ from the medium, but unfortunately both studies used phosphatidylinositol labelling, a method with appreciable limitations (Michell, 1975; Allan & Michell, 1975; Allan *et al.*, 1976), as a secondary measure of the phosphatidylinositol breakdown brought about by the stimulus.

The work described in the present paper was undertaken to determine whether changes in intracellular Na^+ or K^+ concentrations might be involved in the mechanism whereby the activation of muscarinic

cholinergic receptors stimulates phosphatidylinositol breakdown, since it is known that muscarinic cholinergic stimulation causes an increase in cell-surface permeability to these ions in a variety of tissues, including the parotid gland (Selinger *et al.*, 1973; Nishiyama & Pedersen, 1974).

Materials and Methods

Materials were obtained from the same sources as in previous studies (Michell & Jones, 1974; Jafferji & Michell, 1976a).

Six male Wistar rats, weighing 250 g, which had been starved for 18 h, were used in each experiment. The parotid glands were obtained and were chopped and incubated in a modified Krebs–Ringer medium at 37°C as described before (Michell & Jones, 1974; Jones & Michell, 1974, 1975). When all the glands had been obtained, the tissue fragments were recovered by filtration and divided into two approximately equal portions. The first portion was put into a Krebs–Ringer medium of the following composition: NaCl , 112 mM; KCl , 12 mM; MgSO_4 , 1.2 mM; CaCl_2 , 1.9 mM; Na_2EDTA , 0.2 mM; KH_2PO_4 , 1.25 mM; sodium 3-hydroxybutyrate, 5 mM; adjusted to pH 7.4 by gassing with $\text{O}_2 + \text{CO}_2$ (95:5). The second portion was put into a Ca^{2+} -free ion-depleted medium of one of the following compositions: (1) sucrose, 0.32 M; sodium 3-hydroxybutyrate, 5 mM; Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl] ethane-sulphonic acid], 5 mM; Na_2EDTA , 0.2 mM; adjusted to pH 7.4 with Na_2CO_3 ; giving a final Na^+ concentration of about 10 mM and a final K^+ concentration of less than 0.1 mM (as assessed by flame photometry); or (2) sucrose, 0.27 M; Hepes, 30 mM; Tris, 20 mM; Na_2EDTA , 0.2 mM; giving a Na^+ concentration of less than 1 mM and a K^+ concentration of about

10 μ M (again as assessed by flame photometry). After 30 min incubation at 37°C the two batches of tissue were each divided into a number of equal portions and put into fresh media, which were of the same compositions except that for half of each group of tissue samples the media contained 2 mM- (experimental series 1) or 0.2 mM- (experimental series 2) carbamoylcholine chloride. Incubation was continued for a further 15 min, after which the tissue fragments were recovered, and their lipids extracted, separated by chromatography on formaldehyde-treated paper and analysed (Michell & Jones, 1974). The tissue-free filtrates of the ion-depleted media were analysed for Na⁺ and K⁺ by flame photometry. The Na⁺ concentration did not differ from that in the original media and the final concentration of K⁺ was always 1 mM or less. Results are presented as ratios that compare the phosphate content of phosphatidylinositol with the phosphate content of a mixed lipid sample containing the majority of the tissue phospholipids (Jones & Michell, 1974, 1975).

Results and Discussion

The information presented in Table 1 shows that stimulation with carbamoylcholine caused a highly significant decrease in the tissue phosphatidylinositol concentration in all of the situations examined. However, this decrease was slightly less marked in the tissue incubated in the two ion-depleted media than in that incubated in a complete Krebs–Ringer medium. In addition, the phosphatidylinositol content of the samples in the ion-depleted tissue fragments was consistently slightly higher than in the ionic medium,

in both the control and the carbamoylcholine-stimulated tissue samples; the reason for this is not known.

An important consequence of activation of muscarinic cholinergic and α -adrenergic receptors in the parotid gland is an increase in cell-surface permeability to several ions, particularly Ca²⁺, Na⁺ and K⁺, with a high proportion of the current across the membrane carried by the latter two ions (Selinger *et al.*, 1973; Nishiyama & Pedersen, 1974). However, the present results show that carbamoylcholine-stimulated phosphatidylinositol breakdown persists, though probably at a somewhat diminished rate, even when parotid-gland fragments are deprived almost entirely of these three ions for a considerable time. They therefore confirm that the Ca²⁺ influx, which is at least partially responsible for triggering the outward flux of K⁺ across the membrane (Selinger *et al.*, 1973), is not the trigger for phosphatidylinositol breakdown (Jones & Michell, 1975; Oron *et al.*, 1975). They also appear to rule out any such role for Na⁺ or for K⁺.

Agonist-stimulated phosphatidylinositol breakdown therefore appears to be an event that is independent of receptor-stimulated ion fluxes across the surface of the cell. One explanation for this situation might be that phosphatidylinositol breakdown precedes these ion fluxes; it might even be involved in some way in their initiation. This is the point of view expressed in a recently developed hypothesis, which suggests that phosphatidylinositol breakdown has appropriate characteristics for a role in the mechanism of action of muscarinic receptor systems and of other receptor systems that increase cell-surface permeability to Ca²⁺ (Michell, 1975; Michell *et al.*, 1976; Jafferji & Michell, 1976a,b,c).

Table 1. *Carbamoylcholine-stimulated phosphatidylinositol breakdown in parotid-gland fragments in physiological and ion-depleted incubation media*

Conditions for the incubation and analysis of tissue samples, including the compositions of ion-depleted media 1 and 2 are given in the Materials and Methods section. The results presented are means $\pm$ S.E.M. (numbers of tissue samples analysed in parentheses).

	Ratio of phosphate content (phosphatidylinositol/other phospholipids)
Experimental series 1	
Complete Krebs–Ringer medium	0.099 $\pm$ 0.003 (14)
Complete Krebs–Ringer medium plus carbamoylcholine	0.079 $\pm$ 0.002 (13)*
Ca ²⁺ -free ion-depleted medium 1	0.102 $\pm$ 0.002 (13)
Ca ²⁺ -free ion-depleted medium 1 plus carbamoylcholine	0.086 $\pm$ 0.002 (13)*
Experimental series 2	
Complete Krebs–Ringer medium	0.095 $\pm$ 0.003 (8)
Complete Krebs–Ringer medium plus carbamoylcholine	0.079 $\pm$ 0.002 (10)*
Ca ²⁺ -free ion-depleted medium 2	0.105 $\pm$ 0.002 (10)
Ca ²⁺ -free ion-depleted medium 2 plus carbamoylcholine	0.091 $\pm$ 0.001 (12)*

* Significantly different from incubations without carbamoylcholine ($P \leq 0.001$).

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