The relationship of phosphatidylinositol turnover to receptors and calcium-ion channels in rat parotid acinar cells

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(Received 9 July 1980/Accepted 9 October 1980)

To help elucidate the possible role of phosphatidylinositol in the regulation of membrane permeability to Ca^{2+} , the relationship in the rat parotid gland of phosphatidylinositol turnover to hormone receptor binding and to the hormone-mediated increase in K^+ permeability (a Ca^{2+} -dependent phenomenon) was investigated. The concentrations of adrenaline and substance P required to stimulate phosphatidylinositol turnover were found to be similar to those required for the Ca^{2+} -mediated change in K^+ permeability and for ligand binding. However, in the case of muscarinic (cholinergic) receptor stimulation, the phosphatidylinositol response was better correlated to the increase in membrane permeability to Ca^{2+} , as determined by the change in K^+ permeability, than to receptor occupation. Consistent with this relationship between the phosphatidylinositol response and Ca^{2+} -channel activation were results obtained by simultaneous administration of maximal or submaximal concentrations of muscarinic and α -adrenergic agonists. The extent of ^{32}P incorporation when stimulated by maximal concentrations of two agonists did not summate, but, rather, was intermediate between the response of either agonist alone. One interpretation for these observations is that the phosphatidylinositol response may not be related to receptor occupation or activation, but may be involved in the Ca^{2+} -gating mechanism itself.

A number of studies have suggested that phosphoinositides may play a major role in many biological functions. Although phosphoinositides constitute only a small fraction of membrane phospholipids, their presence and metabolism have been related to cellular processes such as changes in Ca^{2+} permeability (Michell *et al.*, 1976; Fain & Berridge, 1979; Berridge & Fain, 1979), K^+ permeability (Abdel-Latif *et al.*, 1977, 1978) and tyrosine hydroxylase activity (Lloyd, 1979).

Hormonal regulation of phosphatidylinositol metabolism was first observed in the pancreas by Hokin & Hokin (1954). They demonstrated that specific hormones, acetylcholine and pancreozymin stimulated the incorporation of ^{32}P into phosphatidylinositol. Since that initial observation, similar responses have been observed in a wide variety of tissues (Michell, 1979).

Recently, a unifying hypothesis has been proposed that relates the turnover of phosphatidyl-

inositol ('the phosphatidylinositol effect') to changes in membrane permeability to Ca^{2+} (Michell, 1975, 1979; Michell *et al.*, 1976). Michell has noted that specific hormonal stimuli in many cases interact with membrane receptors to cause both phosphatidylinositol breakdown and an increased membrane permeability to Ca^{2+} . This observation led to the hypothesis that phosphatidylinositol breakdown was related to activation of surface membrane Ca^{2+} 'channels' or 'gates' (Michell, 1979). In the parotid gland, both a decrease in phosphatidylinositol (Jones & Michell, 1974) and an increase in phosphatidate content (Putney *et al.*, 1980b) have been shown to be produced by hormonal stimuli that also increased membrane permeability to Ca^{2+} . In the parotid, phosphatidylinositol turnover is neither dependent on influx of Ca^{2+} , nor mimicked by the introduction of Ca^{2+} intracellularly with the cationophore A23187 (Rossignol *et al.*, 1974; Jones & Michell, 1974, 1975, 1978; Oron *et al.*, 1975).

To study the role of phosphatidylinositol in Ca^{2+} gating, the rat parotid gland serves as a useful model system. The parotid gland has three types of receptors (peptidergic, cholinergic and α -adrenergic),

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which have been suggested to activate a common Ca^{2+} channel (Marier *et al.*, 1978). All three have been previously shown to stimulate phosphatidylinositol turnover (Jones & Michell, 1974, 1975, 1978). In the present study, we have attempted to exploit this multiple receptor control of phosphatidylinositol turnover in the parotid to better understand the relationship between the phosphatidylinositol effect, receptors and Ca^{2+} -gating mechanisms.

Experimental

Male Sprague-Dawley rats (140–200 g) were anaesthetized with pentobarbitone (50 mg/kg body wt.) and their parotid glands were excised. The glands were finely minced with a razor blade and incubated at 37°C under an atmosphere of 100% O_2 for 5 min in an incubation solution of the following composition (mM): NaCl, 120.0; KCl, 5.0; sodium β -hydroxybutyrate, 5.0; Tris, 20.0; sufficient HCl to adjust the pH to 7.4 at 37°C; no added Ca^{2+} or Mg^{2+} , 0.1 mM-EGTA. Another incubation medium (normal Tris buffer) included both 1.0 mM- Ca^{2+} and 1.5 mM- Mg^{2+} without EGTA. The tissue fragments were resuspended in normal Tris buffer containing 0.5% bovine serum albumin and 1 mg of collagenase/ml. To aid cell dispersion, the tissue fragments were drawn through a pipette (orifice diameter, 3 mm) twice during collagenase incubation. After a 45–60 min incubation in collagenase, the cell suspension was filtered through eight layers of bridal veil on to normal Tris buffer containing 4% bovine serum albumin and centrifuged for 5 min at 200 g. The supernatant was discarded and the pellet was washed twice in Tris buffer containing 4% bovine serum albumin (centrifugation through 4% bovine serum albumin separated cell fragments from dispersed cells and acini). Finally, the cells and acini were resuspended at a concentration of 1.5 mg of protein/ml in normal Tris buffer and allowed to equilibrate for 30 min. At this point, cell viability (usually >95%) was assessed by exclusion of Trypan Blue.

After the 30 min equilibration period, [^{32}P]P_i (10 μM ; 50 $\mu\text{Ci/ml}$) was added to the cell suspension. After 30 min, drugs were added to the samples (or saline for controls). At various times thereafter (usually 30 min), the experiments were terminated by the addition of samples (0.9 ml each) to a mixture of chloroform/methanol (1:2, v/v; 3.4 ml). Lipids were extracted with a modification of the procedure of Bligh & Dyer (1959), as described by Lapetina & Michell (1972).

The phospholipids in the lipid extract were separated by using a modification of the one-dimensional chromatography method reported by Kaulen (1972). Good one-dimensional separation of

phosphatidylinositol from other major phospholipids was achieved with glass plates coated with 0.3 mm of Merck Silica HF containing 0.4% (w/w) $(\text{NH}_4)_2\text{SO}_4$, dried overnight and activated in an oven at 100°C for at least 1 h.

The solvent system that yielded the best separation was chloroform/methanol/acetic acid/water (50:30:8:2.5, by vol.) at 4°C. When atmospheric conditions (i.e. humidity) changed, the water content of the solvent system was modified to optimize separation. By using the chromatographic method described above, good separation of phosphatidylinositol from phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine was achieved.

The phosphatidylinositol spots were scraped from the t.l.c. plates into borosilicate test tubes and digested in HClO_4 solution (0.2 ml) containing ammonium molybdate (Dawson, 1960). Radioactivity was determined by liquid-scintillation spectrometry and P_i was determined with the phosphate reagent described by Itaya & Ui (1966). The protein content of each sample was determined from the cellular precipitate obtained from the interface between the organic and aqueous phases during lipid extraction. The precipitates were solubilized in 3 M-NaOH and analysed for protein by the method of Lowry *et al.* (1951).

Statistical analyses of the data were performed with one-way analysis of variance or Student's *t* test. Only an *F* or *t* statistic greater than those tabulated for *P* = 0.05 was considered significant. Dispersions about the mean represent one standard error of the mean.

Type IV collagenase, bovine serum albumin, acetyl- β -methylcholine (methacholine), adrenaline and the lipid standards (phosphatidylinositol, phosphatidylcholine and phosphatidylserine) were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Substance P was obtained from Peninsula Laboratory, San Carlos, CA, U.S.A. The organic solvents and HClO_4 were reagent-grade chemicals. The [^{32}P]P_i was purchased from Amersham, Arlington Heights, IL, U.S.A., and was essentially carrier-free.

Results

The muscarinic agonist methacholine caused an increase in the incorporation of ^{32}P into both phosphatidylinositol and into phospholipids that ran with the solvent front (neutral lipids, cardiolipin and phosphatidate). The incorporation of ^{32}P into phosphatidylinositol expressed per mg of protein was linear with time from *t* = 0 (time of drug addition) to 60 min (Fig. 1). The phosphatidylinositol response was also calculated on the basis of phosphate content (μg of phosphorus in phosphatidylinositol)

Fig. 1. Time course of ^{32}P incorporation into phosphatidylinositol

The cells were pre-incubated in $10\mu\text{M}$ - ^{32}P P_i for 30 min at 37°C in normal Tris buffer. At $t = 0$, cells designated by open symbols (Δ , $\circ$) were given 0.1 mM -methacholine and those designated by closed symbols ($\blacktriangle$, $\bullet$) were left untreated. The turnover of phosphatidylinositol was examined both on the basis of the incorporation of $^{32}\text{P}/\mu\text{g}$ of phosphorus ($\circ$, $\bullet$) and $^{32}\text{P}/\text{mg}$ of protein (Δ , $\blacktriangle$).

and it appeared that the relative increase in specific radioactivity of phosphatidylinositol was greater than the increase of ^{32}P incorporation into phosphatidylinositol based on cellular protein. The difference between incorporation of ^{32}P expressed per mg of protein or per μg of phosphorus probably resulted from a net decrease in total phosphatidylinositol content. Phosphatidylinositol content decreased by approx. 25% when the cells were incubated with 0.1 mM -methacholine, confirming the original observation by Jones & Michell (1974). The phosphatidylinositol content of control slices was $11.87 \pm 0.48\text{ nmol/mg}$ of protein and for slices incubated for 30 min with 0.1 mM -methacholine the content was $8.74 \pm 0.32\text{ nmol/mg}$ of protein ($P < 0.05$ by paired Student's t test). Subsequent data were derived from specific radioactivity measurements, although qualitatively similar results are

Fig. 2. Cumulative dose-response curves of ^{32}P incorporation/ μg of phosphorus of phosphatidylinositol by substance P, methacholine and adrenaline

The cells were stimulated with adrenaline (Δ), substance P ($\bullet$) and methacholine ($\circ$) for a period of 30 min. The change in the incorporation of ^{32}P into phosphatidylinositol was expressed as the percentage change compared with a maximal response to 0.1 mM -methacholine.

obtained if expressed per mg of protein, especially when data are compared with a standard response such as that obtained with 0.1 M -methacholine.

All three hormones (methacholine, adrenaline and substance P) stimulated incorporation of ^{32}P into phosphatidylinositol. However, substance P and adrenaline caused only 25% and 50% respectively of the ^{32}P incorporation produced by maximal stimulation with methacholine (Fig. 2).

The EC_{50} values (the concentration of hormone required to produce 50% of maximal response) estimated from the dose-response curves of both substance P and adrenaline to produce phosphatidylinositol turnover were comparable with the EC_{50} values for both the increase in membrane permeability to K^+ (Rb^+) produced by these agonists and ligand binding affinity measured directly by using radioactive ligands (Table 1; Strittmatter *et al.*, 1977; Putney *et al.*, 1980a). The increase in membrane permeability to K^+ has been suggested to result from an increase in intracellular Ca^{2+} , which in turn results from Ca^{2+} -channel activation (Selinger *et al.*, 1974). In the case of the muscarinic agonist, the relationship between receptor occupation and the increase in K^+ permeability (an index of Ca^{2+} -channel activation) differed (Putney & Van De Walle, 1980). In the present study, the EC_{50} for the stimulation of phosphatidylinositol turnover produced by methacholine was similar to that for the increase in membrane permeability to K^+ (Table 1; Fig. 3).

To examine further the relationship of the phosphatidylinositol response to hormone receptors, the

Table 1. *EC₅₀ (K⁺ release and phosphatidylinositol response) and K_d (radioligand displacement) values for methacholine, adrenaline and substance P in the rat parotid gland*

Agonist	Receptor	K _d (M)	EC ₅₀ (K ⁺ or ⁸⁶ Rb ⁺) (M)	EC ₅₀ (phosphatidylinositol) (M)
Methacholine	Muscarinic	3 × 10 ^{-6*}	2 × 10 ^{-7*}	2 × 10 ⁻⁷
Adrenaline	α-Adrenergic	2 × 10 ^{-6†}	5 × 10 ^{-6†}	2 × 10 ⁻⁶
Substance P	Substance P	4 × 10 ^{-9‡}	4 × 10 ^{-9‡}	3 × 10 ⁻⁹

* From Putney & Van De Walle (1980).
† Estimated from Fig. 10 of Strittmatter *et al.* (1977).
‡ From Putney *et al.* (1980a).

Fig. 3. *Comparison of methacholine dose-response curves for receptor binding, ⁸⁶Rb efflux and ³²P-incorporation into phosphatidylinositol*

The theoretical binding isotherm (dotted line) was calculated from the K_d (2.76 × 10⁻⁶ M) derived from the inhibition of ³H-QNB binding (Putney & Van De Walle, 1980). The K⁺ efflux (dashed line) was plotted from the experimentally derived K_d of 2.2 × 10⁻⁷ M (Putney and Van De Walle, 1980). The phosphate incorporation/μg of phosphorus in phosphatidylinositol (●) exhibited an EC₅₀ of 2 × 10⁻⁷ M.

effect of simultaneous multiple hormonal activation of ³²P incorporation into phosphatidylinositol was investigated. If the phosphatidylinositol response were associated with the activation of Ca²⁺-channels, one would not expect to observe an additive increase in the phosphatidylinositol effect when the cells were incubated with two agonists. However, if the phosphatidylinositol response were related to receptor binding or activation, the degree of incorporation of ³²P into phosphatidylinositol due to two agonists in combination might reflect the stimulation of the individual responses due to both agonists given alone. Table 2 shows that the simultaneous combination of two agonists employed at maximally effective concentrations failed to produce an increase in incorporation of ³²P greater than that due to the more efficacious agonist given alone. Table 2 shows that the simultaneous combination of two agonists employed at maximally effective concentrations failed to produce an increase

Table 2. *The effect of simultaneous multiple stimulation on the phosphatidylinositol effect by using maximally effective concentrations of methacholine, adrenaline and substance P*

The cells were prepared and incubated with ³²P and the indicated hormone(s) for 30 min as described in the Experimental section. The values are expressed as a percentage of the maximal incorporation of ³²P into phosphatidylinositol obtained by using 0.1 mM-methacholine. The values are expressed as means ± S.E.M. for the numbers of observations shown in parentheses.

	Response (% of maximum)
Agonists alone	
Methacholine (0.1 mM)	100
Adrenaline (0.1 mM)	46.0 ± 3.7* (11)
Substance P (0.1 μM)	24.8 ± 2.3* (8)
Agonists added simultaneously	
Methacholine (0.1 mM)	79.6 ± 3.2* (15)
+ adrenaline (0.1 mM)	
Methacholine (0.1 mM)	90.7 ± 4.1* (7)
+ substance P (0.1 μM)	
Adrenaline (0.1 mM)	48.2 ± 4.4*† (8)
+ substance P (0.1 μM)	

* Significantly less than 100%.
† Not significantly different from 0.1 mM-adrenaline alone.

in incorporation of ³²P greater than that due to the more efficacious agonist given alone. These experiments were repeated with a submaximal concentration of methacholine (0.1 μM), a near maximal concentration of adrenaline (8 μM) and a maximal concentration of substance P (0.1 μM). Since, in the combination, the agonist with the lower efficacy (adrenaline or substance P) is employed at near maximal concentration, the prediction is still summation only if each receptor activates a separate pathway. Again, however, the concomitant addition of either substance P or adrenaline with methacholine failed to produce summation of the phosphatidylinositol responses of the agonists (Table 3).

Table 3. *The effects of simultaneous multiple stimulation on the phosphatidylinositol response, by using sub-maximal concentrations of methacholine*

The protocol was as for Table 2. Results are means $\pm$ S.E.M. for the numbers of observations shown in parentheses.

	Response (% of maximum)
Agonists alone	
Methacholine (0.1 mM)	100
Methacholine (0.2 μM)	30.6 ± 1.0 (3)
Methacholine (0.1 μM)	25.9 ± 5.5 (6)
Adrenaline (8 μM)	37.7 ± 2.3 (8)
Substance P (0.1 μM)	24.8 ± 2.3 (8)
Agonist added simultaneously	
Methacholine (0.2 μM)	$42.0 \pm 2.1^*$ (3)
+ adrenaline (8 μM)	
Methacholine (0.1 μM)	$35.7 \pm 3.8^\dagger$ (6)
+ substance P (0.1 μM)	

* Significantly less than 68.3%, the predicted sum of individual responses to 0.2 μM -methacholine and 8 μM -adrenaline.

† Significantly less than 50.7%, the predicted sum of individual responses to 0.1 μM -methacholine and 0.1 μM -substance P.

Discussion

Michell (1975) has formulated a hypothesis based on the correlation between the ability of hormones to increase both phospholipid metabolism and to increase membrane permeability to Ca^{2+} . He has suggested that the breakdown of phosphatidylinositol may trigger the Ca^{2+} -gating mechanism (Michell, 1979). The theory was supported by the observations that (1) the stimulation of phosphatidylinositol turnover by agonists appears to be independent of extracellular Ca^{2+} (Jones & Michell, 1975, 1978; Oron *et al.*, 1975) and (2) the introduction of Ca^{2+} intracellularly by a bivalent cationophore fails to mimic the actions of hormones in causing phosphatidylinositol turnover (Rossignol *et al.*, 1974; Jones & Michell, 1975; Oron *et al.*, 1975).

The parotid gland is a useful model for studying the role of phosphatidylinositol in Ca^{2+} gating because there are three types of hormone receptors (muscarinic, α -adrenergic and peptidergic). Activation of any of the three types of receptors produces both influx of Ca^{2+} (Marier *et al.*, 1978; Putney *et al.*, 1978) and the phosphatidylinositol response (Michell, 1979; Fig. 2). The experiments in the present study were designed to ascertain whether the phosphatidylinositol response represents a reaction involved in activation of individual receptors, or in the activation of Ca^{2+} channels.

In the cases of both peptidergic and α -adrenergic

stimulation, the isotherm of hormone binding (Strittmatter *et al.*, 1977; Putney *et al.*, 1980a) was similar to the dose-response curves of both the increase in membrane permeability to K^+ (Rb^+) (Strittmatter *et al.*, 1977; Putney *et al.*, 1980a) and incorporation of ^{32}P into phosphatidylinositol (Table 1). However, for the muscarinic receptor, the K_d for receptor occupation by methacholine was approx. 10-fold greater than the EC_{50} for Ca^{2+} gating (K^+ release), presumably due to a 10-fold excess (i.e. 'spare') of muscarinic receptors (Putney & Van De Walle, 1980). Fig. 3 shows that when the data are scaled to the same maximum, the dose-response curve for the phosphatidylinositol effect more closely approximates to the Ca^{2+} -gating curve (i.e. K^+ release) than the receptor-binding curve. Therefore, a close relationship between phospholipid metabolism and activation of the ' Ca^{2+} -channel' would seem likely. Since incorporation of ^{32}P into phosphatidylinositol measures resynthesis of phosphatidylinositol rather than the suspected primary event, phosphatidylinositol breakdown, it could be argued that the discrepancy between the phosphatidylinositol-labelling curve and receptor-binding curve reflects a lack of close association between labelling and breakdown. Although it is difficult to eliminate conclusively this possibility without direct breakdown measurements, there are some points that suggest that the assumption of association between labelling and breakdown is not unreasonable. First, the close agreement between phosphatidylinositol labelling and K^+ release would, if the breakdown and labelling are not associated, be attributed to coincidence. Secondly, the agreement between phosphatidylinositol labelling and K^+ release is consistent with subsequent results of summation experiments. Thirdly, in the liver where comparison of breakdown and labelling due to vasopressin was made, a close association was observed (Kirk *et al.*, 1981).

Another strategy of the present study was to examine the phosphatidylinositol turnover produced by multiple simultaneous hormonal stimulation. Previously, a similar experimental protocol was employed to assess the relationship of the three receptors to produce the influx of Ca^{2+} (Marier *et al.*, 1978). In those experiments, simultaneous hormonal stimulation failed to cause an additive change in membrane permeability to Ca^{2+} , as interpreted from monitoring the Ca^{2+} -dependent efflux of $^{86}\text{Rb}^+$ (Marier *et al.*, 1978), or from direct measurement of $^{45}\text{Ca}^{2+}$ influx (Putney *et al.*, 1978). These results support the theory that the three receptors stimulate the same population of Ca^{2+} channels. In other words, all three receptor mechanisms appear to converge at or impinge on a common Ca^{2+} -channel mechanism. If multiple stimulation of the phosphatidylinositol response were to follow a similar pattern as that for Ca^{2+} influx (or $^{86}\text{Rb}^+$

efflux), the incorporation of ^{32}P into phosphatidylinositol would not be additive on application of agonists in combination. However, if the phosphatidylinositol effect was associated with either a receptor interaction or some step before Ca^{2+} -channel activation, the stimulation of each receptor would be independent of the others and might therefore produce an additive effect. Simultaneous stimulation, however, failed to produce an increase in phosphatidylinositol turnover that was greater than that of the more efficacious response (Table 1). This failure to observe an additive effect was probably not due to the saturation of some later step involved in phosphatidylinositol labelling resulting from supramaximal stimulation by methacholine (0.1 mM), since both adrenaline and substance P (each of which produced less phosphatidylinositol turnover than a maximal concentration of methacholine) also did not produce an additive effect when combined. Additionally, submaximal stimulation by methacholine (Table 2) produced similar results to those observed at a maximal effective concentration of methacholine. In conclusion, concomitant stimulation by two agonists did not produce an additive effect, which suggests that simultaneous stimulation of the phosphatidylinositol-effect through multiple receptors converges at some step subsequent to receptor occupancy.

These data, therefore, suggest that phosphatidylinositol turnover may be a reaction more closely associated with Ca^{2+} gating, rather than a reaction directly involved in receptor activation or occupation. The precise role played by phosphatidylinositol in Ca^{2+} gating, however, is not yet clearly established. It has recently been suggested that it may be the net formation of phosphatidic acid that is most important in activating Ca^{2+} transport (Salmon & Honeyman, 1980; Putney *et al.*, 1980b). Obviously, continued investigation in this area will be necessary to understand more fully the interrelationships of hormone receptors, phospholipid metabolism and Ca^{2+} -gating mechanisms.

These studies were supported in part by grants from the National Institutes of Health (U.S.A.) nos. DE-04067 and GM-07176.

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