Regulation of phosphatidate synthesis by secretagogues in parotid acinar cells

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(Received 15 December 1981/Accepted 16 February 1982)

The metabolism of phosphatidate in rat parotid acinar cells was investigated, particularly with regard to the actions of agonists known to act by mobilizing Ca^{2+} . When cells were incubated in medium containing $10\mu\text{M}$ - $[^{32}\text{P}]\text{P}_i$, phosphatidate was rapidly labelled, approaching an apparent steady-state with a half-time of approx. 20 min. Methacholine provoked a more than doubling of phosphatidate radioactivity, which was reversed by the muscarinic antagonist atropine. These results suggest that phosphatidate labels to near steady-state rapidly and that in cells prelabelled for 60 min the increase in radioactivity induced by agonists probably reflects net synthesis rather than an increase in specific radioactivity. Phosphatidate synthesis in response to methacholine was rapid and occurred, within the resolution of a few seconds, with no measurable latency. Adrenaline and substance P also stimulated phosphatidate synthesis but both agonists were less efficacious than methacholine. A Ca^{2+} ionophore, ionomycin, did not provoke phosphatidate synthesis. By using a protocol that eliminates the receptor-regulated Ca^{2+} pool, it was demonstrated that methacholine-induced phosphatidate formation does not come about as a consequence of Ca^{2+} influx nor of Ca^{2+} release. These results indicate that the phosphatidate synthesis response has characteristics compatible with its previously suggested role as a primary mediator of membrane Ca^{2+} -gating.

Activation of a variety of surface-membrane receptors leads to an enhanced turnover of phosphatidylinositol (Michell, 1975), an effect first described for the action of acetylcholine on the exocrine pancreas by Hokin & Hokin (1954). The precise role for this turnover in the response of the cell to agonists is not known. Michell (1975, 1979) has suggested that breakdown of phosphatidylinositol or its phosphorylated derivatives (Kirk *et al.*, 1981) may be the initial event leading to the enhanced turnover, and may play some role in the mechanism by which receptors mobilize cellular Ca^{2+} . The evidence for this hypothesis is the ubiquitous association of phosphatidylinositol turnover with receptors that utilize Ca^{2+} as second messenger, and the demonstration (for most systems) that phosphatidylinositol turnover or breakdown is not a Ca^{2+} -mediated event (Michell, 1979).

More recently, it has been suggested that synthesis of phosphatidate, believed to occur secondary to phosphoinositide breakdown, may play an important role in Ca^{2+} -mobilization, perhaps by functioning as a Ca^{2+} -ionophore (Salmon & Honeyman, 1980; Putney *et al.*, 1980). However, there are few data on the characteristics of phosphatidate synthesis as affected by receptor activation. This report summarizes recent findings on the effects in parotid acinar cells of secretagogues on phosphatidate synthesis. Specifically, these experiments examined the time course of the phosphatidate synthesis due to secretagogues, and the role of Ca^{2+} in the phosphatidate synthesis response.

Experimental

Parotid glands were removed from anaesthetized (sodium pentobarbitone) male Sprague–Dawley rats (140–200 g) and dispersed acinar cells were prepared as previously described (Putney *et al.*, 1978). The cells were suspended at a concentration of

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1–2 mg of protein/ml in a Ringer medium of the following composition (mM): NaCl, 120; KCl, 5.0; $MgCl_2$, 1.0; Tris, 20; sodium β -hydroxybutyrate, 5.0; and 0.5% bovine serum albumin. The media were titrated with HCl (about 10 mM final Cl^- concentration) to a pH of 7.4 at 37°C. The gas phase was 100% O_2 . In some experiments, ionized Ca^{2+} was reduced to $<1 \mu M$ by the addition of 2 mM-EGTA (neutralized with NaOH).

Cells were equilibrated with the standard medium for 20 min, at which time $10 \mu M$ - $[^{32}P]P_i$ was added as the neutral sodium salt. The specific radioactivity of the $[^{32}P]P_i$ was adjusted so that the $10 \mu M$ concentration provided a radioactivity concentration of about 5 $\mu Ci/ml$. At various times thereafter drugs were added to the media and portions of the cell suspension were taken for analysis. The portions (0.6 ml) were rapidly homogenized in 2.25 ml of chloroform/methanol (1:2, v/v) and the phospholipids were extracted by the method of Bligh & Dyer (1959) as modified by Lapetina & Michell (1972). The phosphatidate was separated from other radioactive phospholipids by t.l.c. in one dimension and visually detected by autoradiography. The method employs 10 cm plates coated with 0.25 mm silica gel 60 (EM reagents). The chromatograms were developed twice in the same dimension in a solvent system of chloroform/methanol/acetic acid/water; in the first development the proportions were 40:10:10:1 (by vol.) and in the second development, they were 120:46:19:3. Typical R_f values for the phospholipids containing measurable radioactivity were: phosphatidate, 0.85; phosphatidylethanolamine (trace), 0.65; phosphatidylinositol, 0.35; phosphatidylcholine, 0.18; polyphosphoinositides, origin.

The area corresponding to phosphatidate was scraped into a scintillation vial, 5 ml of water was added and the radioactivity was quantified from Čerenkov radiation in a liquid-scintillation counter. The extent of labelling is generally expressed as a percentage of some control value, usually just before drug additions. Experiments were generally repeated three to four times and means $\pm$ S.E.M. are given. All observed effects of drugs etc. are based on demonstrated statistical significance ($P < 0.05$), usually by analysis of variance.

The enzymes used for cell dispersion, acetyl- β -methylcholine (methacholine), adrenaline, propranolol, isoprenaline, phentolamine and phospholipid standards were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Substance P was purchased from Peninsula Laboratories, San Carlos, CA, U.S.A. Ionomycin was kindly donated by Mr. S. J. Lucania of the Squibb Institute for Medical Research, Princeton, NJ, U.S.A. The $[^{32}P]P_i$ was purchased from New England Nuclear, Boston, MA, U.S.A.

Results

The rate of incorporation of $[^{32}P]P_i$ into phosphatidate appears to be first-order with a half-time of about 20 min (Fig. 1), and with labelling being essentially complete in 1 h. This suggests rapid turnover of the phosphate headgroup of phosphatidate. The incorporation of $[^{32}P]P_i$ into ATP may be rate-limiting when intact cells are used. Evidence that the hormone-sensitive pool of phosphatidate (or ATP) is labelled to near steady-state under these conditions is given in Fig. 2. In these experiments, cells were pre-equilibrated with the $[^{32}P]P_i$ containing medium for 30 or 60 min, at which time 0.1 mM-methacholine was added to the suspension. After a further 5 min, a sample was taken for analysis and $10 \mu M$ -atropine was added to the suspension. After an additional 10 min, a second sample was taken. The results of these experiments, summarized in Fig. 2, show that methacholine causes a more than doubling of radioactivity in phosphatidate, and that subsequent addition of atropine, a muscarinic-receptor-blocking drug, essentially reverses this response. The pattern obtained was similar whether cells were pre-incubated with $[^{32}P]P_i$ for 30 or 60 min. Also, the relative

Fig. 1. Time course of incorporation of $[^{32}P]P_i$ into phosphatidate

(a) Shows radioactivity in phosphatidate of rat parotid acinar cells as a function of incubation time in medium containing $10 \mu M$ - $[^{32}P]P_i$. Data are expressed as percentages of 90 min values. Results are means of four experiments; S.E.M. values averaged less than 50% of the means. (b) Shows a semi-logarithmic plot of data from the first 30 min illustrating apparent first-order kinetics.

Fig. 2. Relative effect of methacholine and subsequent effect of atropine on phosphatidate radioactivity after 30 min or 60 min prelabelling

After 30 or 60 min prelabelling in [32 P] P_i , cells were stimulated with 0.1 mM-methacholine (M) for 5 min, and then 10 μ M-atropine (A) was added to the suspension. Radioactivity in phosphatidate was determined 5 min after the addition of methacholine and 10 min after the addition of atropine and were in each case expressed as a percentage of the value obtained just before the addition of methacholine. Results are means of four experiments $\pm$ S.E.M. (represented by the bars).

Fig. 3. Comparison of time course of labelling of phosphatidate (O) and phosphatidylinositol (●) in response to methacholine

Parotid cells were incubated in medium containing 10 μ M- $[^{32}$ P] P_i for 60 min, at which time 0.1 mM-methacholine was added. Radioactivity in phosphatidate and phosphatidylinositol was calculated as a percentage of the value obtained from a sample taken just before drug addition. Also included are data for the first 30s for phosphatidate in unstimulated controls (Δ). Results are means from four to five experiments $\pm$ S.E.M. (represented by bars).

increase in radioactivity in phosphatidate (2.25-fold in the 60 min group) was similar to that reported previously for the increase in total content of phosphatidate (2.28-fold; Putney *et al.*, 1981). For subsequent experiments, cells were pre-incubated in medium containing [32 P] P_i for 60 min before addition of agonists. These results support the contention that the rapid labelling of phosphatidate indicates rapid equilibration of phosphatidate with its immediate precursor (ATP) and indicates that relative changes in phosphatidate radioactivity reflect changes in cellular content of phosphatidate.

The time course of labelling of phosphatidate compared with labelling of phosphatidylinositol is shown in Fig. 3. Phosphatidate labelling precedes phosphatidylinositol labelling by about 2 min. The first sample taken, 12 s after methacholine, was significantly elevated in phosphatidate content compared with samples taken at $t=0$, or compared with samples taken concomitantly from unstimulated controls (shown for the first 30 s only). Regression analysis of the phosphatidate labelling data for the first 30 s revealed no significant deviation of the

intercept of the y -axis from $t=0$, the resolution of this analysis being of the order of 2–3 s.

Agonists acting through the Ca^{2+} -mobilization pathway were compared as to their abilities to activate phosphatidate synthesis; the results are shown in Fig. 4. Neither adrenaline nor substance P stimulated phosphatidate synthesis to an extent equal to methacholine. The time course of response to methacholine and adrenaline was similar. Substance P activated phosphatidate synthesis at a rate similar to methacholine for the first 2 min, but phosphatidate labelling ceased to increase thereafter such that the net increases after 5–10 min was similar to that for adrenaline. In three similar experiments (results not shown) the β -adrenoceptor agonist, isoprenaline, did not significantly elevate phosphatidate levels.

Fig. 4. Time course for net synthesis of phosphatidate in response to various agonists

The protocol was as for Figs. 2 and 3 except that the agonists used were: ●, none (control); ○, 0.1 mM-methacholine; △, 0.1 mM-adrenaline; □, 0.1 μM-substance P. Results are means ± S.E.M. from six experiments.

The bivalent cationophore, ionomycin, was also examined as to its ability to activate phosphatidate synthesis. The concentration of ionomycin used, 2.67 μM, activates Ca^{2+} influx of a magnitude similar to that for methacholine in the parotid, as indicated by ^{86}Rb efflux (Poggioli *et al.*, 1982). In four experiments this concentration of ionomycin, however, had no effect on phosphatidate or phosphatidylinositol labelling.

The possibility that phosphatidate labelling might arise as a result of receptor-mediated mobilization of Ca^{2+} in the parotid acinar cell was investigated in experiments summarized in Fig. 5. Labelling of phosphatidylinositol and phosphatidate in response to methacholine was determined in the presence of 1 mM or lowered (<1 μM) extracellular Ca^{2+} . No significant effect of lowering external Ca^{2+} on the pattern of phosphatidylinositol or phosphatidate labelling was detected in five separate experiments (not shown).

The failure of lowering external Ca^{2+} to <1 μM to affect significantly phosphatidate synthesis essentially rules out Ca^{2+} influx as a mediator of the response, but leaves open the possibility that intracellular Ca^{2+} release, known to occur in the absence of external Ca^{2+} , might trigger the effect. To examine this possibility, use was made of a phenomenon previously described as 'cross-receptor

Fig. 5. Effect of sequential stimuli in low- Ca^{2+} medium on phosphatidate synthesis

Cells were labelled for 60 min in 10 μM- $^{32}\text{P}[\text{P}]_i$. At 5 min before the addition of drugs, Ca^{2+} was reduced to <1 μM by the addition of 2 mM-EGTA. Symbols: ○, control; ●, 0.1 mM-adrenaline from 0 to 30 min, 0.1 mM-phenolamine from 10 to 30 min, 0.1 mM-methacholine from 20 to 30 min. Results are means ± S.E.M. (represented by the bars) for four experiments.

inactivation' (Putney, 1977). It has previously been shown that, in the absence of extracellular Ca^{2+} , only one cellular Ca^{2+} release response can be elicited, regardless of the nature of the receptor activated. This is due to the fact that all three of the Ca^{2+} -mobilizing receptors release a common cellular pool of Ca^{2+} , which can only be refilled from the extracellular space (Putney *et al.*, 1981). Thus, in experiments summarized in Fig. 5, parotid cells were stimulated with adrenaline in a medium containing <1 μM- Ca^{2+} , and a stimulation of phosphatidate synthesis occurs. The stimulus is removed (pharmacologically) with 100 μM-phenolamine, resulting in a return of phosphatidate to the control level. Subsequent stimulation with methacholine, which now cannot activate Ca^{2+} -release due to the 'cross-receptor inactivation' discussed above, causes an immediate rise in cellular [phosphatidate].

Discussion

These findings confirm results from a number of previous studies, and also provide new information on receptor regulation of phospholipid metabolism in the parotid acinar cell. One surprising finding was the rapidity of phosphatidate labelling and results, such as those in Figs. 2 and 5, which suggest that after a 30–60 min pre-labelling period, phosphatidate specific radioactivity is probably changed very little by stimulation. This means that the conventional 'phosphatidylinositol-effect', which is an enhanced $^{32}\text{P}[\text{P}]_i$ labelling of phosphatidylinositol by stimuli, probably does not come about simply because of an increased radioactivity in phosphatidate secondary to diacylglycerol generation. Rather, in the parotid, this must reflect an enhanced

rate of synthesis of phosphatidylinositol from phosphatidate. It is difficult to explain this in terms of a simple mass-action effect, since phosphatidate levels increase less than 3-fold in response to methacholine (Fig. 2), whereas this same agonist provokes an approximate 8-fold increase in the rate of phosphatidylinositol labelling (Weiss & Putney, 1981). This may mean that the concentration of phosphatidate in a specific cellular pool could determine the rate of phosphatidylinositol synthesis or possibly that phosphatidate with the specific fatty acid composition likely to arise from the phosphatidylinositol cycle (1-stearoyl, 2-arachidonyl) is channelled to resynthesis of phosphatidylinositol more rapidly than other molecular species of phosphatidate.

One purpose of this investigation was to determine whether phosphatidate synthesis has properties consistent with its suggested role as a primary mediator of Ca^{2+} influx in the parotid gland (Putney *et al.*, 1980). One requirement for such a mediator would be that its synthesis would be rapidly activated. Electrophysiological measurements (Roberts & Petersen, 1978) suggest that parotid ion fluxes are activated by neurotransmitters with a latency in the order of tenths of seconds. It is unlikely that biochemical measurements of phospholipids can, with present technology, achieve this resolution. Still, the data in Fig. 3 show that there is at least no measurable latency, within a resolution of a few seconds, for the phosphatidate response. Relevant to this result are the findings of Lapetina & Cuatrecasas (1979) that in platelets phosphatidate synthesis is also apparently an immediate response to thrombin stimulation, and precedes the onset of secretion.

Another important property of the phosphatidate synthesis response involves the finding that phosphatidate synthesis does not appear to arise as a consequence of Ca^{2+} influx or release. That Ca^{2+} influx does not mediate phosphatidylinositol breakdown or labelling in the parotid has been clearly demonstrated in previous studies (Jones & Michell, 1975, 1976), and the data here on phosphatidylinositol labelling confirm these findings. Although one could predict from the assumed close relationship between phosphatidylinositol breakdown and phosphatidate synthesis that phosphatidate formation would be similarly independent of Ca^{2+} influx, the data reported here (Fig. 5) demonstrate this directly. Thus phosphatidate synthesis is not activated by artificially introducing Ca^{2+} into the parotid cell with the bivalent cationophore ionomycin. This ionophore is considerably more efficacious in the parotid than ionophore A23187, and is capable of mobilizing Ca^{2+} to an extent similar to receptor-active agonists (Poggioli *et al.*, 1982).

Phosphatidate synthesis in response to metha-

choline is unaltered when experiments are carried out in a medium containing $<1\mu\text{M-Ca}^{2+}$. Under these conditions, however, agonists such as methacholine are known to provoke transient responses (about 2 min in duration) resulting from mobilization of a cellular Ca^{2+} pool (Putney, 1977). Thus the possibility exists that phosphatidate synthesis could come about as a result of this Ca^{2+} release when essentially no Ca^{2+} is present externally. This possibility is excluded, however, by the results of experiments summarized in Fig. 5. In these experiments, cells were activated by adrenaline and subsequently, after α -receptor blockade by phentolamine, activated by methacholine. Throughout the experiment the medium contained $<1\mu\text{M-Ca}^{2+}$. Both the first (adrenaline) and second (methacholine) stimulus resulted in a substantial and significant synthesis of phosphatidate. In the case of the second stimulus (methacholine), it has been previously shown that under these conditions no cellular Ca^{2+} release occurs (Putney, 1977; Haddas *et al.*, 1979). This is due to the fact that the three receptors present in the parotid gland release the same pool of Ca^{2+} , which can only refill from the extracellular space (Putney *et al.*, 1981). Thus, after the stimulus with adrenaline, the receptor-regulated Ca^{2+} pool has been emptied and in the absence of external Ca^{2+} cannot refill when phentolamine is added. Thus when methacholine is added, no Ca^{2+} release occurs, and transient responses usually seen, such as K^{+} release, are absent (Putney *et al.*, 1981). The fact that methacholine is capable of stimulating phosphatidate synthesis under these conditions shows that this response is mediated neither by Ca^{2+} influx nor by Ca^{2+} release.

In conclusion, data from the present study on regulation of phosphatidate synthesis in parotid acinar cells are consistent with the hypothesis that phosphatidate synthesis may provide the critical link between receptor activation and Ca^{2+} -gating (Salmon & Honeyman, 1980; Putney *et al.*, 1980). Agonists activating all three Ca^{2+} -mobilizing receptors provoke phosphatidate synthesis; this synthesis has been shown to occur rapidly and with no measurable latency. The response is not mimicked by a Ca^{2+} ionophore and can be shown to result neither from Ca^{2+} influx nor from Ca^{2+} release. This indicates that phosphatidate synthesis is not itself a consequence of Ca^{2+} mobilization and could, as previously proposed, be formed as a direct result of receptor activation and subsequently function in mediating the receptor-activated Ca^{2+} influx.

Note added in proof (Received 29 March 1982)

A paper has recently appeared with results similar to some of those reported here and with similar conclusions (Miller & Kowal, 1981).

These studies were supported in part by a grant (no. DE-05764) from the National Institutes of Health (U.S.A.).

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