Subcellular distribution of agonist-stimulated phosphatidylinositol synthesis in 1321 N1 astrocytoma cells

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In an inositol-depleted 1321 N1 astrocytoma cell line, propranolol at 0.5 mM concentration and carbachol in the presence of Li^+ induce a large increase (30–60-fold) in the amount of CMP-phosphatidate, the lipid substrate of PtdIns synthase. The actions of both agents on CMP-phosphatidate accumulation were reversed by co-incubation with 1 mM inositol. In cells grown in the presence of 40 μM inositol the propranolol- and carbachol-mediated CMP-phosphatidate accumulation was much smaller (2–4-fold). Propranolol- and carbachol-mediated increases in CMP-phosphatidate accumulation were at least additive in both inositol-replete and -depleted cells. The subcellular distribution of accumulated CMP-phosphatidate was investigated by sucrose-density-gradient centrifugation of a lysate of inositol-depleted cells. There were two coincident peaks of carbachol-stimulated [^{3}H]CMP-phosphatidate and PtdIns synthase activity, respectively. The first peak of accumulated [^{3}H]CMP-phosphatidate and PtdIns synthase activity is characteristic of a 'light vesicle' fraction, since it sediments at sucrose densities similar to that of endocytosed ^{125}I-transferrin. The later peak, containing both carbachol-stimulated [^{3}H]CMP-phosphatidate and PtdIns synthase activity, has a distribution in the gradient that is similar to NADPH–cytochrome *c* reductase activity, an endoplasmic-reticulum marker. By contrast, propranolol-stimulated [^{3}H]CMP-phosphatidate accumulates in membranes which sediment as a single peak corresponding to the endoplasmic-reticulum marker. These observations suggest that agonist-stimulated PtdIns synthesis occurs in the endoplasmic reticulum and in at least one additional membrane compartment which is insensitive to propranolol, an inhibitor of endoplasmic-reticulum phosphatidate phosphohydrolase.

INTRODUCTION

Receptor-stimulated PtdIns(4,5)P_2 hydrolysis is a ubiquitous signal-transduction mechanism which generates the intracellular second messengers, diacylglycerol and Ins(1,4,5)P_3 (Hirasawa and Nishizuka, 1985; Berridge and Irvine, 1989). The replenishment of agonist-sensitive phosphoinositides during continued hormonal stimulation requires the efficient recycling of diacylglycerol and the inositol moiety of Ins(1,4,5)P_3. These recycling reactions culminate in the combination of CMP-phosphatidate and inositol to give PtdIns, a reaction catalysed by CMP-phosphatidate:inositol phosphatidyltransferase (PtdIns synthase; Hokin-Neaverson et al., 1977). Recycling of the inositol moiety of Ins(1,4,5)P_3 is blocked by Li^+ ions at the level of inositol polyphosphate 1-phosphomonoesterase and inositol monophosphate phosphomonoesterase (Hallcher and Sherman, 1980; Majerus et al., 1988), an effect that may explain the molecular basis of the drug's therapeutic utility in the treatment of manic depression (Berridge et al., 1982, 1989; Sillence and Downes, 1992).

Although it is self-evident that hormone-stimulated PtdIns(4,5)P_2 hydrolysis is initiated in the plasma membranes of cells, whether the complete inositol phospholipid metabolic cycle takes place in a single membrane compartment is currently disputed. Metabolic turnover studies providing evidence both for and against the existence of a discrete hormone-sensitive pool of inositol phospholipids have been reported (Monaco, 1982, 1987; Michell et al., 1988). Alternatively, it has been suggested that PtdIns and phosphatidate could shuttle between the plasma membrane and endoplasmic reticulum (Michell, 1975) via phospholipid transfer proteins or vesicular mechanisms (Van Paridon et al., 1987; Sleight and Abanto, 1989). In an attempt to resolve these issues, Gershengorn and his colleagues have sought and characterized a PtdIns synthase activity in membrane fractions enriched in plasma membranes of GH_3 pituitary cells (Imai and Gershengorn, 1987; Cubitt and Gershengorn, 1989). However, others have failed to detect a plasma-membrane PtdIns synthase; for example, this enzyme appeared to be exclusively located in the endoplasmic reticulum of C6 rat glioma cells and N1E-115 murine neuroblastoma cells (Morris et al., 1990).

Here we show that, in 1321 N1 astrocytoma cells, CMP-phosphatidate accumulates in an agonist-dependent fashion in the presence of Li^+ and in an agonist-independent fashion in the presence of 0.5 mM propranolol, an amphipathic cationic drug that acts largely by inhibiting endoplasmic-reticulum phosphatidate phosphohydrolase (Jamal et al., 1991). As CMP-phosphatidate is the immediate precursor of PtdIns, we have attempted to exploit the dramatic accumulation of this liponucleotide which occurs in inositol-depleted cells as a marker of the cellular compartment(s) in which agonist-stimulated PtdIns synthesis takes place.

MATERIALS AND METHODS

Materials

DL-Propranolol, carbamoylcholine chloride (carbachol) and [5-^{3}H]cytidine were obtained from Sigma. NADPH, cytochrome *c* and concanavalin A were obtained from Boehringer Mannheim. Trypsin/EDTA was from Flow Laboratories. Plastic 80 cm^2 flasks and 1.5 cm^2 multiwells were from NUNC. AnalaR sucrose was obtained from BDH.

Abbreviation used: PtdCho, phosphatidylcholine.

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Cell culture

Human astrocytoma cells (1321 N1) were cultured as described previously (Meeker and Harden, 1982). Cells were partially inositol-depleted by a similar protocol to that of Stephens et al. (1989). Confluent cell monolayers were washed once with inositol-free Dulbecco's modified Eagle's medium. The cells were dissociated with trypsin/EDTA. Dissociated cells were either diluted into Dulbecco's modified Eagle's *myo*-inositol-free medium supplemented with 5% dialysed fetal-calf serum or containing 40 μM *myo*-inositol supplemented with 5% fetal-calf serum. Cells were plated at a density of 50000 cells/cm^2 in an atmosphere of air/CO_2 (19:1) at 37 °C in a humidified incubator. Confluent flasks (80 cm^2, plastic) or multiwells (1.5 cm^2, plastic) of cells were routinely used for experiments 5 or 6 days after seeding (2 days after the last medium change).

Subcellular fractionation

For monitoring the distribution of [^{3}H]CMP-phosphatidate, the flask was incubated in 10 ml of Hepes-buffered Krebs medium (pH 7.4, 37 °C, of the following composition: NaCl, 114 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; Hepes, 25 mM; $CaCl_2$, 1.3 mM) containing 11 mM glucose and 20 μCi of [^{3}H]cytidine. The flasks of cells were labelled for 1 h at 37 °C. The flasks were then incubated with 1 mM carbachol, 5 mM LiCl or 0.5 mM propranolol for 30 min in 100 μl of buffer. Each flask was then washed with 2 × 10 ml and then incubated in 10 ml of ice-cold Hepes-buffered Krebs medium of the same composition as above but including 0.1 mM $MnSO_4$ and 0.5 mg/ml concanavalin A and omitting glucose (pH 7.4, 4 °C). After 5 min the flasks were washed with 10 mM Hepes (pH 7.4, 4 °C) and incubated in 10 ml of this buffer for 20 min on ice. Incubation in this hypo-osmotic buffer causes the cells to swell, but not to burst. The buffer was then removed, and the cells were lysed and harvested by scraping the flask with a rubber policeman. The lysate (~ 1 ml) was transferred to a microfuge tube and centrifuged for 30 s at 12400 *g* to remove the unlysed cells and nuclei. The supernatant was then layered over a sucrose gradient.

Linear continuous sucrose gradients were formed with a Pharmacia f.p.l.c. system. Gradients consisted of a 1 ml cushion of 5% sucrose on top of a 9 ml linear gradient of 24–60% sucrose, both in 10 mM Hepes, pH 7.4. Centrifugation was for 60 min at 35000 rev./min (217000 $g_{max.}$) in a Beckman SW 40 Ti rotor in a Beckman L8-70 refrigerated ultracentrifuge at 4 °C. The continuous gradients were fractionated by injecting 70% (w/v) sucrose into the bottom of the centrifuge tube and then collecting fractions (0.6 ml) from the top with a Dyeguard (Diagnostica) fractionator connected to a fraction collector.

Enzyme assays

PtdIns synthase was assayed at 37 °C for 30 min in the following medium: Hepes (pH 7.8, 20 °C), 20 mM; $MgCl_2$, 10 mM; CMP-phosphatidate, 0.1 mM; *myo*-inositol, 1 mM; *myo*-[^{3}H]inositol, 10 μCi; and 1–4 μg of membrane protein. Incubations were stopped by addition of 1 ml of 6% $HClO_4$, vortex-mixed and spun down in a bench-top microfuge for 20 min. The supernatant was removed and [^{3}H]PtdIns was extracted as follows: 750 μl of methanol/chloroform/0.1 M HCl (80:40:1, by vol.) was added to the acid precipitate, followed by a further 250 μl of chloroform and 450 μl of 0.1 M HCl. The contents were vortex-mixed thoroughly. A 400 μl portion of the lower phase was mixed with 10 ml of Pharmacia Optiphase 'High safe 3' scintillant and counted for radioactivity after leaving in the dark for several hours to decrease chemiluminescence. Over 95% of the chloroform-soluble radioactivity was found in a compound with the expected mobility of PtdIns on t.l.c. when this assay was applied to A431-cell membranes and turkey erythrocyte ghosts (C. Vaziri and C. P. D. Downes, unpublished work).

5′-Nucleotidase (Avruch and Wallach, 1971) and adenylate cylase (Evans, 1980) activity were used as plasma-membrane markers. NADPH–cytochrome *c* reductase was assayed as a marker for endoplasmic reticulum (Evans, 1980); however, this enzyme also occurs in cytosol (Morand and Kent, 1986). Early fractions which were significantly contaminated with cytosol were diluted 2-fold with ice-cold buffer (Hepes, pH 7.4) and the membranes were collected by centrifugation in a Beckman TL-100 ultracentrifuge (80000 rev./min in a TLA 100.2 rotor at 4 °C for 30 min). NADPH–cytochrome *c* reductase was measured as follows. Incubations in a total volume of 1 ml contained approx. 40 μg of membrane protein, NADPH (0.1 mM), KCN (0.1 mM), cytochrome *c* (0.1 mM) and KH_2PO_4 buffer (50 mM, pH 7.5). The membranes were added last to initiate the reaction, which was monitored at room temperature at 550 nm in a Cecil 6000 dual-beam spectrophotometer in the kinetics mode.

Cell-associated ^{125}I-transferrin was used to identify endosomal membranes (Sandvig et al., 1987). Briefly, ^{125}I-transferrin (2 × 8.6 μg; sp. radioactivity 14000 c.p.m./μg of protein) was added to two 80 cm^2 flasks. The flasks contained 10 ml of Hepes-buffered Krebs medium of the same composition as described above. To one of the flasks 200 μg/ml unlabelled transferrin was added to determine non-specific binding. The flasks were then incubated at 37 °C for 30 min and then lysed and fractionated as described above. Fractions from the sucrose gradients were then counted for ^{125}I-transferrin with a γ-radiation counter. Non-specific binding was less than 1% of the total.

Protein was measured by the Peterson (1977) modification of the Lowry method, with BSA as a standard.

Measurement of CMP-phosphatidate

1321 N1 cells were grown in multiwell plates under inositol-depleted and -replete conditions as described. Cells were incubated in 300 μl of Hepes-buffered Krebs medium (as above) labelled with 0.1–1 μCi of [^{3}H]cytidine at 37 °C for 1 h. LiCl, carbachol and propranolol were added in 10 μl of buffer to final concentrations of 5, 1 and 0.5 mM respectively. Incubations were terminated by addition of 1 ml of 6% $HClO_4$. The acidified medium was aspirated off and the cells were scraped in a further 500 μl of 6% $HClO_4$. The precipitates were spun down in a bench-top microcentrifuge, resuspended in 1 ml of 6% $HClO_4$ and spun down again. The supernatant was aspirated and [^{3}H]CMP-phosphatidate was extracted from the washed pellets and counted for radioactivity as described above for [^{3}H]PtdIns. In subcellular-fractionation experiments, 600 μl of 6% $HClO_4$ was added to each 600 μl fraction. After vortex-mixing [^{3}H]CMP-phosphatidate was extracted as above.

RESULTS

[^{3}H]CMP-phosphatidate labelling in inositol-replete and -depleted 1321 N1 cells

The cellular accumulation of CMP-phosphatidate, which can be conveniently monitored by the incorporation of [^{3}H]cytidine into a chloroform-soluble fraction (Downes and Stone, 1986; Godfrey, 1989) is a sensitive indicator of an inositol-deficient state in some cells. In GH_3 and WRK-1 cells, however, inositol deficiency appears not to be accompanied by the detectable accumulation of CMP-phosphatidate (Drummond and Raeburn, 1984; Rodriguez et al., 1987; Monaco and Adelson, 1991). As

Table 1 Carbachol and propranolol enhancement of [^{3}H]CMP-phosphatidate labelling in 1321 N1 astrocytomas grown in 40 μM inositol

Inositol-replete cells were grown to confluence in 1.5 cm^2 multiwells and were labelled with 1 μCi of [^{3}H]cytidine per well for 1 h at 37 °C. Li^+ (final concn. 5 mM) was added 10 min before addition of agonist. The final concentrations of carbachol and propranolol were 1 mM and 0.5 mM. Incubations were stopped with 750 μl of 6% $HClO_4$. Precipitates were pooled from three wells and processed together. [^{3}H]CMP-phosphatidate was extracted as described. Data represent the total amount of [^{3}H]CMP-phosphatidate from three wells and are expressed as d.p.m. (means ± S.E.M. for the numbers of experiments given in parentheses).

	Radioactivity (d.p.m.) in CMP-phosphatidate	
Incubation	30 min	60 min
Control	184 ± 19 (4)	227 ± 20 (4)
Control/Li^+	171 ± 19 (4)	245 ± 43 (4)
Carbachol	305 ± 28 (2)	342 ± 19 (2)
Carbachol/Li^+	432 ± 58 (4)	633 ± 141 (4)
Propranolol	766 ± 123 (4)	1086 ± 96 (4)
Propranolol/Li^+	679 ± 164 (4)	1005 ± 176 (4)
Carbachol/Li^+/propranolol	1445 ± 113 (4)	2012 ± 349 (4)
Carbachol/propranolol	1216 ± 238 (2)	1734 ± 212 (2)

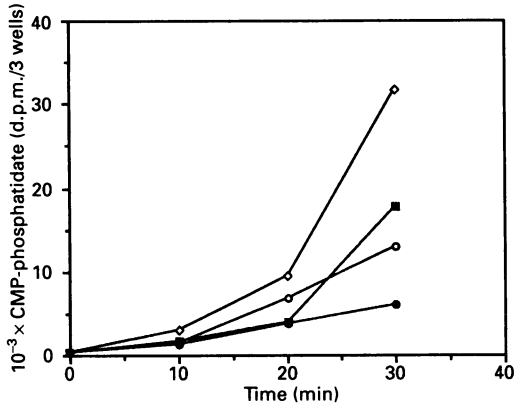

Figure 1 Time courses of carbachol- and propranolol-mediated increases in [^{3}H]CMP-phosphatidate labelling in inositol-depleted 1321 N1 cells

Multiwells of inositol-depleted astrocytoma cells were labelled with 1 μCi of [^{3}H]cytidine. Incubations were stopped with 6% $HClO_4$, and three walls were pooled and processed together. Data are expressed as total [^{3}H]CMP-phosphatidate/3 wells and are from one experiment respresentative of three. Control values at 0 min were 301 ± 9 d.p.m., rising to 374 ± 74 d.p.m. after 30 min. Addition of carbachol alone did not stimulate CMP-phosphatidate accumulation. Symbols: ○, 1 mM carbachol, 5 mM Li^+; ■, 0.5 mM propranolol; ●, 0.5 mM propranolol, 1 mM carbachol; ◇, 0.5 mM propranolol, 1 mM carbachol and 5 mM Li^+.

shown below, CMP-phosphatidate clearly accumulates during agonist stimulation of both inositol-replete and -depleted 1321 N1 cells.

Inositol-replete cells

In 1321 N1 cells grown in the presence of 40 μM inositol, carbachol induced a 2-fold increase in [^{3}H]CMP-phosphatidate labelling over the first 30 min (Table 1). This effect was not dependent on, or significantly enhanced by, Li^+, suggesting that the carbachol-mediated increase was not due to a decrease in cellular inositol supply. However, Li^+ increased the carbachol-stimulated [^{3}H]CMP-phosphatidate labelling by 2-fold at 60 min of stimulation, suggesting that inositol levels can be decreased significantly over prolonged incubation periods. Propranolol

Table 2 Inositol reversal of carbachol- and propranolol-mediated [^{3}H]CMP-phosphatidate accumulation of inositol-depleted 1321 N1 cells

Multiwells were labelled with 0.1 μCi of [^{3}H]cytidine. Li^+ (final concn. 5 mM), propranolol (0.5 mM) and carbachol (1 mM) were added as described. Inositol was added to a final concentration of 1 mM at the same time as propranolol or carbachol. Incubations were stopped after 30 min with $HClO_4$ and processed separately. Data are from triplicate incubations and are expressed as d.p.m. (means ± S.E.M. from one experiment which was representative of 2–5 experiments).

Incubation	Radioactivity (d.p.m.)
Control	201 ± 32
Control/Li^+	139 ± 3
Propranolol	2290 ± 101
Propranolol/inositol	310 ± 19
Carbachol/Li^+	1343 ± 208
Carbachol/Li^+/inositol	194 ± 19
Carbachol	179 ± 6

addition caused a 4-fold accumulation of CMP-phosphatidate over the first 30 min. The propranolol-mediated response was not significantly enhanced by the presence of Li^+. Table 1 also shows that, in inositol-replete cells, the carbachol- and propranolol-mediated increases in [^{3}H]CMP-phosphatidate labelling are additive, but not obviously synergistic.

Inositol-depleted cells

In inositol-depleted cells, carbachol-stimulated accumulation of [^{3}H]CMP-phosphatidate was dependent on the presence of Li^+. Figure 1 shows that addition of carbachol and Li^+ leads to a large (30–40-fold) increase in CMP-phosphatidate. This appears to occur, not because of any dramatic decline in the intracellular inositol content, but because the small pool of intracellular inositol is insufficient to support the required PtdIns synthase activity (I. H. Batty and C. P. Downes, unpublished work). In the absence of Li^+, however, inositol phosphates produced by the action of agonist-stimulated phospholipase C are rapidly dephosphorylated, leading to a large increase in inositol concentration (I. H. Batty and C. P. Downes, unpublished work), which prevents the accumulation of CMP-phosphatidate. Figure 1 also shows that addition of propranolol at 0.5 mM concentration to inositol-depleted 1321 N1 cells caused a 40–60-fold increase in [^{3}H]CMP-phosphatidate labelling, as it does in rat sciatic nerve (Zhu and Eichberg, 1990). This effect is not dependent on the presence of Li^+ and is reported to be due predominantly to the inhibition of endoplasmic-reticulum phosphatidate phosphohydrolase (Jamal et al., 1991; Zhu and Eichberg, 1990).

In contrast with inositol-replete cells, the combined response to carbachol/Li^+ and propranolol in inositol-depleted cells is greater than the sum of the individual responses. This might be expected if propranolol and carbachol/Li^+ exert mechanistically distinct effects on CMP-phosphatidate accumulation in the same membrane compartment. A further noteworthy feature of the CMP-phosphatidate response is that, in the absence of Li^+, carbachol actually inhibits the propranolol response (Figure 1). This is likely to be due to the large increase in inositol concentration that accompanies carbachol stimulation of the inositol-depleted cells, as noted above.

Table 2 shows that the increase in [^{3}H]CMP-phosphatidate labelling induced by the addition of carbachol in the presence of Li^+, and that induced by the addition of propranolol in inositol-depleted cells, were decreased substantially in the presence of

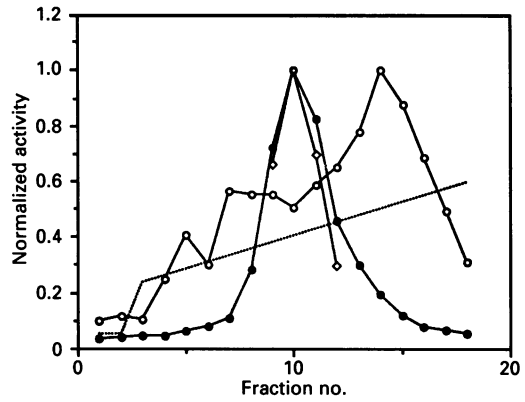

Figure 2 Sucrose-gradient distribution of marker enzymes

Inositol-depleted cells from 80 cm^2 confluent flasks were hypotonically lysed, and a post-nuclear supernatant (1 ml) was layered on a linear 9 ml 24–60% (w/v) sucrose gradient with a 1 ml 5%-sucrose cushion. After spinning at 35000 rev./min in a SW-40 rotor at 4 °C for 1 h, 0.6 ml fractions were taken as assayed. Fraction 1 refers to the top of the gradient. Adenylate cyclase was measured at 30 °C for 10 min. 5′-Nucleotidase incubations were at 37 °C for 15 min. NADPH–cytochrome *c* reductase was measured as described in the Materials and methods section. Adenylate cyclase and 5′-nucleotidase were assayed from the same gradient in one experiment. NADPH–cytochrome *c* reductase was assayed on a separate gradient, and the results shown are typical of two experiments. Data are normalized such that peak activity is 1.00, except for sucrose, where 0.6 is equal to 60% sucrose. Symbols: ◇, adenylate cyclase; ●, 5′-nucleotidase; ○, NADPH–cytochrome *c* reductase; ----, sucrose.

1 mM *myo*-inositol. This suggests that such changes in labelling of [^{3}H]CMP-phosphatidate reflect a net accumulation of this liponucleotide due to limitation of PtdIns synthase activity by the small pool of intracellular inositol. The degree of [^{3}H]CMP-phosphatidate accumulation appears smaller in these experiments, because the wells were labelled with less [^{3}H]cytidine than for the experiment depicted in Figure 1 (0.1 rather than 1 μCi). For unknown reasons, the assay background was always proportionately greater when the more economic labelling regime was used.

Subcellular distribution of carbachol- and propranolol-stimulated [^{3}H]CMP-phosphatidate accumulation in inositol-depleted cells

Fractionation of plasma membrane and endoplasmic reticulum

The procedure followed for the fractionation of 1321 N1 cells involved incubation of the cells with concanavalin A, followed by hypotonic lysis and layering the lysate on a sucrose gradient (Lutton et al., 1979; Waldo et al., 1983; Hoover and Toews, 1989). At pH > 7, in the presence of Mn^{2+} and Ca^{2+}, concanavalin A binds to mannose and glucose residues on the cell surface and leads to the stabilization of the plasma membrane. After mild homogenization, such as the hypotonic-swelling/cell-scraping method used here, the plasma membranes migrate more uniformly at denser fractions in the sucrose gradient. Figure 2 shows data for plasma-membrane and endoplasmic-reticulum markers from one experiment that is representative of 20 experiments. 5′-Nucleotidase activity co-sedimented with fluoride-stimulated adenylate cyclase, giving a symmetrical peak at 40–50% sucrose. This result is similar to that reported previously in these cells for the plasma-membrane marker (Lutton et al., 1979; Waldo et al., 1983; Hoover and Toews, 1989). The distribution of the endoplasmic-reticulum marker NADPH–cytochrome *c* reductase was similar to that found for hypotonically lysed GH_3 cells (Imai and Gershengorn, 1987). Consistent with the distribution of NADPH–cytochrome *c* reductase activity, glucose-6-phosphatase immunoreactivity, which should be solely found in the endoplasmic reticulum, was confined to the denser fractions of a discontinuous gradient (results not shown).

Despite the use of a similar procedure for the fractionation of 1321 N1 cells, the distribution of the endoplasmic-reticulum marker in Figure 2 is different from that reported by Waldo et al. (1983), who assayed glucose-6-phosphatase to monitor the distribution of the endoplasmic reticulum. They reported that most of the activity sedimented at the top of the gradient. It is not clear, however, whether they managed to distinguish between glucose-6-phosphatase activity and non-specific phosphatase activity. We found glucose-6-phosphatase activity that did not sediment with glucose-6-phosphatase immunoreactivity, suggesting the presence of non-specific phosphatase(s) (results not shown).

Initially, attempts were made to assay marker enzymes, PtdIns synthase and [^{3}H]CMP-phosphatidate in the same gradients. However, this proved impractical because of the large amounts of cellular material required, especially for the relatively insensitive NADPH–cytochrome *c* reductase assay. Generally, 5′-nucleotidase was assayed across all the gradients analysed, and the distribution of PtdIns synthase and CMP-phosphatidate was compared directly. Data arising from the same or parallel gradients are indicated in Figure legends. In gradients where [^{3}H]CMP-phosphatidate was measured, 75 μl from each 600 μl fraction was taken to assay the plasma-membrane marker. Early fractions were contaminated with [^{3}H]cytidine, which interfered with the 5′-nucleotidase assay.

The specific activity of NADPH–cytochrome *c* reductase in the post-nuclear lysate was 0.21 ± 0.07 μmol/min per μg of pelletable protein, which increased 3-fold to 0.65 μmol/min per μg in the peak fraction; 61 ± 5% of the activity was recovered on the gradient. The specific activity of 5′-nucleotidase in the post-nuclear lysate was 55 ± 11 pmol/min per μg of pelletable protein, which increased more than 10-fold to 573 ± 140 pmol/min per μg in the peak fraction; 50 ± 1.5% of the total post-nuclear-lysate activity was recovered on the gradient. Of the total [^{3}H]CMP-phosphatidate in the homogenate, 79 ± 9% was recovered from the low-speed spin and 51 ± 7% of this was recovered on the gradient.

Subcellular distribution of propranolol-stimulated [^{3}H]CMP-phosphatidate

Figure 3 shows the distribution of propranolol-stimulated [^{3}H]CMP-phosphatidate accumulation, NADPH–cytochrome *c* reductase and the plasma-membrane marker, 5′-nucleotidase. There is a close correlation between the endoplasmic-reticulum marker and that of propranolol-stimulated [^{3}H]CMP-phosphatidate distribution, suggesting that propranolol stimulates accumulation of the liponucleotide specifically in the endoplasmic reticulum. This is consistent with the observation that, although there appear to be distinct phosphatidate phosphohydrolases in endoplasmic reticulum and plasma membrane (Day and Yeaman, 1992), the latter is preferentially inhibited by 0.5 mM propranolol (Jamal et al., 1991). The possibility that an amphipathic cation such as propranolol could lead to a detergent effect that alters the sedimentation profile of [^{3}H]CMP-phosphatidate was addressed by comparing the sedimentation properties of the plasma-membrane marker 5′-nucleotidase in the presence and absence of propranolol. Figure 3 shows that the sedimentation properties are similar to that found in Figure 2, so this possibility appears unlikely.

Subcellular distribution of PtdIns-synthesis markers

Figure 4 shows the sucrose-gradient distribution of [^{3}H]CMP-

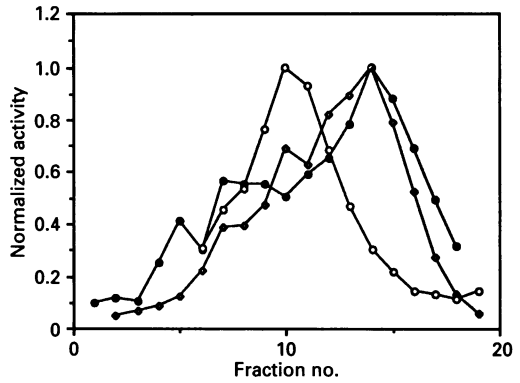

Figure 3 Sucrose-gradient distribution of propranolol-stimulated [^{3}H]CMP-phosphatidate accumulation, NADPH–cytochrome *c* reductase and 5′-nucleotidase activity

Cells from 80 cm^2 flasks were lysed and fractionated as described for Figure 2. Fraction 1 refers to the top of the gradient. NADPH–cytochrome *c* reductase was used as the endoplasmic-reticulum marker. From co-determination of the distribution of [^{3}H]CMP-phosphatidate distribution and the plasma-membrane marker, 75 μl of each fraction was taken and assayed for 5′-nucleotidase activity. Cytosolic [^{3}H]cytidine interfered with the assay of early fractions. Data are expressed with the peak activity normalized to 1.00. Data are respresentative of at least two experiments. Symbols: ○, 5′-nucleotidase; ●, NADPH–cytochrome *c* reductase; ◇, propranolol.

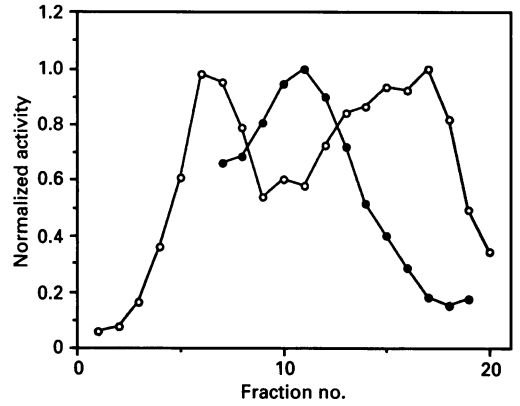

Figure 4 Sucrose-gradient distribution of carbachol-stimulated [^{3}H]CMP-phosphatidate accumulation in the presence of Li$^+$

Inositol-depleted cells in 80 cm^2 flasks were labelled with 20 μCi of [^{3}H]cytidine for 1 h and then stimulated with 1 mM carbachol in the presence of 5 mM Li$^+$ or 0.5 mM propranolol for 30 min. Flasks were washed with ice-cold Hepes Krebs–Ringer buffer containing 0.5 mg/ml concanavalin A, followed by lysis buffer. The post-nuclear lysate was layered on the sucrose gradient. After centrifugation, 0.6 ml fractions were taken and 75 μl from each fraction was used to assay the plasma-membrane marker before acid precipitation and for extraction of [^{3}H]CMP-phosphatidate as described in the Materials and methods section. 5′-Nucleotidase was used as the plasma-membrane marker; [^{3}H]cytidine interferes with the 5′-nucleotidase assay in the early fractions. [^{3}H]CMP-phosphatidate accumulation in the presence of 5 mM Li$^+$ was 190 $\pm$ 20 d.p.m./fraction, which in the presence of carbachol or propanolol increased typically to 5000–10000 d.p.m. in the peak fraction. The data are expressed such that the peak of activity is normalized to 1.00. The data are representative of three experiments. Symbols: ●, 5′-nucleotidase; ○, CMP-phosphatidate accumulated in the presence of 1 mM carbachol and 5 mM Li$^+$.

phosphatidate which accumulated after 30 min of stimulation with carbachol in the presence of Li$^+$. A time of 30 min was chosen because it was near the plateau for [^{3}H]CMP-phosphatidate accumulation under the conditions employed (Figure 1). It is clear that carbachol in the presence of Li$^+$ leads to an accumulation of [^{3}H]CMP-phosphatidate which is con-

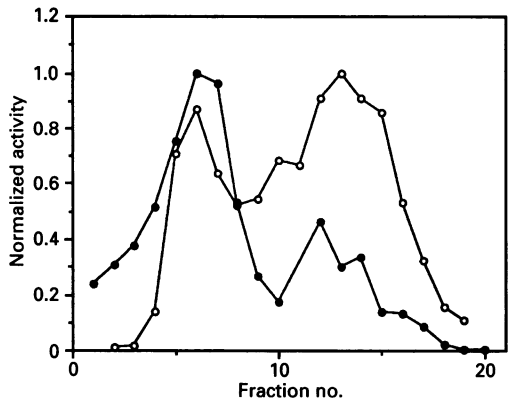

Figure 5 Sucrose-gradient distribution of PtdIns synthase activity and ^{125}I-transferrin-labelled endosomes

Fraction 1 refers to the top of the gradient. PtdIns synthase activity was measured by the forward reaction in the presence of 0.1% Triton and 1 mM inositol. For the endosome marker, 8.6 μg of ^{125}I-transferrin was added to each flask (sp. radioactivity 14000 c.p.m./ng) and incubated at 37 °C for 30 min as described. Non-specific binding was determined in the presence of 200 μg/ml unlabelled transferrin, and was not greater than 1% of the total. PtdIns synthase distribution is representative of three separate experiments. Results for endocytosed ^{125}I-transferrin is from one experiment representative of three. Data are normalized such that the peak activity is 1.00. Symbols: ●, ^{125}I-transferrin; ○, PtdIns synthase.

centrated in two main peaks on the gradient. The first peak occurs at 30–36% sucrose, and the second peak at 50–56% sucrose. Figure 4 also shows the distribution of 5′-nucleotidase in the same gradient. The activity is centred around a peak at 40–50% sucrose. Early fractions could not be assayed for 5′-nucleotidase, as cytosolic [^{3}H]cytidine interfered with the assay. The data clearly show that most of the carbachol-mediated [^{3}H]CMP-phosphatidate accumulation is not associated with the plasma membrane. The second peak of carbachol-stimulated [^{3}H]CMP-phosphatidate accumulation reproducibly sedimented at similar densities to that of propranolol-stimulated CMP-phosphatidate and of NADPH–cytochrome *c* reductase.

The subcellular distribution of PtdIns synthase activity is shown in Figure 5; clearly it follows that of its substrate, [^{3}H]CMP-phosphatidate. An early peak is seen around 30–36% sucrose, before the plasma-membrane marker, and a later peak at around 50–56% sucrose, after the plasma-membrane marker. This result is consistent with the observation that carbachol-stimulated [^{3}H]CMP-phosphatidate accumulation is reversible by inositol. Imai and Gershengorn (1987) reported a significant portion of the PtdIns synthase activity co-migrating with the plasma-membrane marker. In contrast, our results show that a significant portion of PtdIns synthase activity is associated with a lighter fraction distinct from the plasma membrane. The possibility that a minor portion of the PtdIns synthase activity co-migrates with the plasma membrane cannot be excluded, however.

Figure 5 shows that the endosomal marker ^{125}I-transferrin migrates at similar densities (30–33% sucrose) to the first peak of PtdIns synthase activity and carbachol-induced [^{3}H]CMP-phosphatidate accumulation. However, Golgi membranes also sediment early in the gradient (Waldo et al., 1983). It is clear that a portion of the PtdIns synthase activity and carbachol-stimulated [^{3}H]CMP-phosphatidate migrates at 50–56% sucrose, similar to that of the endoplasmic-reticulum marker. This result is similar to that observed by Imai and Gershengorn (1987) in GH_3 cells; they confirmed the dual distribution of PtdIns synthase (Figure 5) by reporting distinct kinetic parameters in the two

subcellular locations. We were unable to assess kinetic properties of the PtdIns synthase activities monitored on the gradient, because there was insufficient enzyme activity for more detailed experiments.

DISCUSSION

The results clearly show that propranolol, at 0.5 mM concentration, causes the accumulation of CMP-phosphatidate in cells grown in the presence of 40 μM inositol and in cells depleted in inositol. The main site of propranolol's action is reported to be the inhibition of phosphatidate phosphohydrolase (Zhu and Eichberg, 1990). Phosphatidate phosphohydrolase is an important branch point in phospholipid synthesis. Inhibition of this enzyme leads to the complete inhibition of phosphatidylcholine (PtdCho) and phosphatidylethanolamine synthesis and the accumulation of phosphatidic acid which is then channelled towards PtdIns and phosphatidylglycerol synthesis (Eichberg et al., 1979). In inositol-depleted cells, PtdIns synthesis is blocked at the level of PtdIns synthase, and so its substrate, CMP-phosphatidate, accumulates. In liver, 0.5 mM propanolol inhibits mainly endoplasmic-reticulum phosphatidate phosphohydrolase (Jamal et al., 1991). The endoplasmic reticulum is particularly active in phospholipid metabolism (Terce et al., 1992). Our results are in agreement with these observations in that propranolol-stimulated accumulation of CMP-phosphatidate appears to be restricted to the endoplasmic reticulum.

In contrast, carbachol induces an increase in [^{3}H]CMP-phosphatidate accumulation as a consequence of PtdIns(4,5)P_2 hydrolysis. In inositol-depleted cells in the presence of Li^+, the accumulation of this precursor for PtdIns synthesis is greatly enhanced. Li^+ blocks the dephosphorylation reactions that recycle Ins(1,4,5)P_3 to inositol which is required for PtdIns synthesis, thus inducing the accumulation of [^{3}H]CMP-phosphatidate. Addition of carbachol and Li^+ to inositol-depleted cells decreases the level of cellular PtdIns by at least 50%, but this effect occurs with little measurable change in the already low intracellular inositol concentration (I. H. Batty and C. P. Downes, unpublished work). In the absence of Li^+, there is a large increase in inositol concentration which largely prevents the rise in CMP-phosphatidate. The substantial accumulation of CMP-phosphatidate which occurs in response to carbachol and Li^+ suggests that the enhanced resynthesis of PtdIns which accompanies agonist-stimulated inositol lipid breakdown largely reflects an increased metabolic flux between diacylglycerol and CMP-phosphatidate rather than activation of PtdIns synthase itself. This may in turn reflect the increased production of diacylglycerol by phospholipase C, or it may involve regulation of diacylglycerol kinase and/or CMP-phosphatidate synthase. This contradicts Cubitt and Gershengorn's (1989) suggestion that relief of product inhibition of PtdIns synthase underlies agonist-stimulated PtdIns resynthesis, but makes better sense, since the PtdIns synthase step is thermodynamically neutral, whereas the reactions catalysed by CMP-phosphatidate synthase and diacylglycerol kinase are thermodynamically irreversible committed steps.

Our data show that carbachol-mediated accumulation of [^{3}H]CMP-phosphatidate occurs mainly in the endoplasmic reticulum and a 'light vesicle' fraction. The observation that propranolol and carbachol/Li^+ exert synergistic effects on CMP-phosphatidate accumulation in inositol-depleted cells suggests that these stimuli may act in part on a common pool of lipid intermediates, presumably those in the endoplasmic reticulum. However, the 'light vesicle' CMP-phosphatidate indicates that agonist stimulation involves a substantial pool of lipid intermediates that are not in the endoplasmic reticulum. We cannot be specific about what the 'light vesicle' fraction contains, although it is probably associated with either an endosomal or a Golgi compartment or both. Such a compartment could have some association with the plasma membrane where receptor-mediated hydrolysis of PtdIns(4,5)P_2 occurs.

Lipids and lipid-metabolizing enzymes localized in 'light vesicle' fractions are not without precedent in the literature. It is noteworthy that Campbell et al. (1985) reported phosphatidylinositol 4-kinase in coated vesicles. Also, Kinney and Carman (1990) noted the accumulation of both CMP-phosphatidate synthase and PtdIns synthase in post-Golgi-apparatus secretory vesicles in the *sec6-4* mutant of the yeast *Saccharomyces cerevisiae*. Glycolipids have been found to be internalized via endosomes in the same way as receptors (Kok et al., 1989). In one report, PtdCho recycling between the plasma membrane and intracellular compartments occurred via a 'light vesicle' fraction, although the intracellular compartments labelled depended on the cell type (Sleight and Abanto, 1989).

Harden et al. (1985) have suggested that internalized receptors in 1321 N1 cells may retain the ability to couple to G-proteins, raising the possibility that receptor-mediated hydrolysis of PtdIns(4,5)P_2 could occur in an endosome compartment. However, Thompson and Fisher (1991) showed that, in SK-N-SH cells, muscarinic receptors have to return to the cell surface to re-initiate PtdIns(4,5)P_2 hydrolysis. The proposition of a vesicular compartment which is inactive as regards PtdIns(4,5)P_2 hydrolysis, but is capable of resynthesising PtdIns and perhaps phosphorylating it to PtdIns(4,5)P_2, could help to explain evidence for a discrete hormone-sensitive pool of PtdIns (Koreh and Monaco, 1986).

The above model would be consistent with the observation that the species pattern of PtdIns(4,5)P_2 does not change during agonist stimulation (Augert et al., 1989). Our results are consistent with this model if the 'light vesicle' fraction which contains PtdIns synthase and [^{3}H]CMP-phosphatidate is derived from the plasma membrane. However, this cannot be the whole story, since, at least under conditions of inositol depletion, a portion of the [^{3}H]CMP-phosphatidate that accumulates in the presence of carbachol and Li^+ is associated with the endoplasmic reticulum. This leads to the problem of how a plasma-membrane event can lead to the activation of PtdIns synthesis in the endoplasmic reticulum. One possibility is that the diacylglycerol or phosphatidic acid produced from PtdIns(4,5)P_2 hydrolysis are transported via lipid-transfer proteins or vesicles to the endoplasmic reticulum, with PtdIns presumably returning by a similar mechanism (Michell, 1975). Another possibility is that the second-messenger products of agonist-mediated hydrolysis may lead to a phosphorylation event which activates a controlling point in PtdIns biosynthesis. However, agonist-mediated phosphatidate accumulation shows little receptor reserve, which would be expected if such a mechanism was responsible (Cubitt et al., 1990). Also, Monaco and Adelson (1991) have shown that the continued presence of agonist is not required for and does not enhance the resynthesis of PtdIns.

As noted above, Cubitt and Gershengorn (1989) have proposed that the enhancement of PtdIns synthesis is due to relief of product inhibition of PtdIns synthase, but this cannot explain the accumulation of CMP-phosphatidate observed in our experiments. Perhaps a fall in membrane PtdIns content instead activates CMP-phosphatidate synthase and/or diacylglycerol kinase. Such a mechanism would require that a decrease in plasma-membrane PtdIns leads to a similar fall in the PtdIns content of the endoplasmic reticulum (Hokin-Neaverson, 1977; Kirk et al., 1981). This communication may involve the

PtdIns/PtdCho phospholipid-exchange protein (Van Paridon et al., 1987) or intracellular vesicles (Sleight and Abanto, 1989).

The above scenario for the activation of PtdIns synthesis is similar to that proposed for the activation of PtdCho synthesis. PtdCho synthesis is regulated by a number of stimuli which can be mimicked by exposure to exogenously added phospholipase C (Terce et al., 1988). Stimulation of PtdCho synthesis is almost always accompanied by translocation and activation of cytidylyltransferase, which catalyses the committed step in PtdCho synthesis (Terce et al., 1992). In the case of activation of PtdCho synthesis by addition of extracellular phospholipase C, a depletion of plasma-membrane PtdCho or an increase in the level of diacylglycerol leads to an enhancement of the rate of PtdCho biosynthesis in the endoplasmic reticulum. This is proposed to occur after depletion of endoplasmic-reticulum PtdCho (Jamil et al., 1990) and/or an increase in diacylglycerol, which has been proposed to gain access to the endoplasmic reticulum by a facilitated process (Pagano and Longmuir, 1985). Diacylglycerol has been shown to cause translocation of cytidylyltransferase, which catalyses one of the committed steps in PtdCho biosynthesis, to the membrane (Kolesnick and Hemer, 1990). Diacylglycerol is perhaps an attractive candidate for controlling phospholipid biosynthesis, since evidence suggests that the diacylglycerol derived from inositol phospholipid hydrolysis is efficiently re-incorporated into PtdIns in some cells (Hokin and Hokin, 1964; Kirk et al., 1981; Augert et al., 1989). Membrane-bound diacylglycerol kinase activity is specific for the 2-arachidonoyl species derived from inositol phospholipid hydrolysis (MacDonald et al., 1988), and thus could be the first step in committing inositol-phospholipid-derived diacylglycerol to PtdIns resynthesis. As with PtdIns transport, transport of plasma-membrane PtdCho or diacylglycerol to the endoplasmic reticulum may occur via a phospholipid-exchange protein or vesicular transport mechanisms (Pagano and Longmuir, 1985; Van Paridon et al., 1987; Sleight and Abanto, 1989).

In conclusion, we have shown that muscarinic cholinergic stimulation of both inositol-replete and inositol-depleted 1321 N1 cells leads to detectable accumulation of the PtdIns synthase substrate, CMP-phosphatidate. This suggests that agonist-stimulated resynthesis of PtdIns reflects, at least in part, enhanced metabolic flux from diacylglycerol, but whether this involves direct regulation of key enzymes of lipid metabolism or simply increased substrate availability has not been addressed. Very large increases in CMP-phosphatidate were observed in inositol-depleted cells, allowing an assessment of this lipid's subcellular distribution. Since much of the CMP-phosphatidate which accumulated in response to carbachol and Li^+ appeared to be present in the endoplasmic reticulum, there is a requirement for a means of communicating acute changes in lipid composition between membrane compartments, as suggested previously (Michell, 1975; Kirk et al., 1981). However, the occurrence of agonist-stimulated, but not propranolol-stimulated, CMP-phosphatidate in a light-vesicle fraction which contained little plasma membrane adds a further complexity. Studies are currently underway to characterize the CMP-phosphatidate-enriched light-vesicle fraction, which may shed new light on the nature of agonist-sensitive inositol lipid pools.

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