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Enhanced Synthesis *de novo* of Phosphatidylinositol in Lymphocytes Treated with Cationic Amphiphilic Drugs

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1. A variety of amphiphilic cations caused very large increases in the rates of incorporation of P_i and glycerol into phosphatidylinositol in pig mesenteric small lymphocytes. This synthesis *de novo* of phosphatidylinositol led to a doubling of the phosphatidylinositol concentration in the cells within 3.5 h. 2. The increase in synthesis of phosphatidylinositol labelled with [^{3}H]- or [^{14}C]-glycerol was matched by an approximately equivalent decrease in incorporation of glycerol into phosphatidylcholine, phosphatidylethanolamine and triacylglycerol. 3. Amphilic cations which produced these effects included, in order of decreasing effectiveness, trifluoperazine (half-maximal effect at about 70 μM) > chlorpromazine $\approx$ promethazine $\approx$ imipramine > cinchocaine > amethocaine $\approx$ cetyltrimethylammonium > fenfluramine > amphetamine > 2-phenethylamine > cocaine $\approx$ procaine; the most effective compounds were those with the largest and most hydrophobic non-polar substituents. 4. The response to cations was not changed by varying the extracellular Ca^{2+} concentration in the range 10 nM–1 mM. 5. The active amphiphilic cations interacted with anionic phospholipids causing aggregation of aqueous dispersions and/or changes in chromatographic behaviour. 6. These results indicate that amphiphilic cations redirect glycerolipid synthesis *de novo*, probably owing to inhibition of phosphatidate phosphohydrolase, so that phosphatidylinositol synthesis is increased at the expense of other glycerolipids.

Many cells exposed to extracellular stimuli exhibit changes in the metabolism of phosphatidylinositol and sometimes of phosphatidate. In most of these, including lymphocytes treated with mitogenic lectins such as phytohaemagglutinin, this modified metabolism appears to consist of stimulated phosphatidylinositol breakdown followed by resynthesis (see Lapetina & Michell, 1973*a*; Michell, 1975, for reviews).

There have also been a considerable number of reports of modifications to the metabolism of phosphatidylinositol and phosphatidate in a variety of tissues treated with amphiphilic drugs which possess an ionizable amine function, such as chlorpromazine and other phenothiazine tranquillizers (Magee *et al.*, 1963; Magee & Rossiter, 1963; Onaya & Solomon, 1969), imipramine (Brossard & Quastel, 1963), azacyclonol (Magee *et al.*, 1963), cinchocaine and other local anaesthetics (Salway & Hughes, 1972; Hughes & Salway, 1973; Eichberg *et al.*, 1973*a*,*b*; Eichberg & Hauser, 1974), morphine (Mulé, 1967, 1971), levorphanol (Dole & Simon, 1974) and propranolol (Stein & Hales, 1972; Eichberg *et al.*, 1973*b*; Levey *et al.*, 1969).

The relationship between these effects of amphiphilic amines and the stimulated phosphatidylinositol turnover which occurs in cells exposed to 'physiological' stimuli such as phytohaemagglutinin has never been clarified, but there have been suggestions that the two phenomena might be results of two different types of changes in the lipid metabolism of cells (Magee *et al.*, 1963; Eichberg *et al.*, 1973*a*,*b*; Eichberg & Hauser 1974). In particular, it was found that in the pineal gland enhanced labelling only of phosphatidylinositol occurred in response to a physiological stimulus (noradrenaline), but that cinchocaine or propranolol provoked enhanced labelling of several lipids, including phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and CDP-diacylglycerol (Eichberg *et al.*, 1973*b*; Eichberg & Hauser, 1974). The experiments reported in the present paper were aimed at clarification of the relationship between the effects of these two types of compounds, both of which stimulate $^{32}P_i$ incorporation into phosphatidylinositol in several types of cell. Such clarification appeared to be needed because of certain recent observations which might have suggested a link between the two sets of observations. First, an enzyme which may be responsible for phosphatidylinositol breakdown in phytohaemagglutinin-treated lymphocytes has a requirement for a low concentration of Ca^{2+} ions and might be controlled by changes in the intracellular Ca^{2+} concentration (Allan & Michell,

1974*b*). Second, lymphocyte activation shows a requirement for extracellular Ca^{2+} and can be triggered by the entry of Ca^{2+} into cells (Maino *et al.*, 1974). Third, cinchocaine and chlorpromazine, both of which are amphiphilic amines, can, in circumstances where they cause a rise in Ca^{2+} concentration, activate phosphatidylinositol breakdown catalysed by the soluble lymphocyte enzyme (Allan & Michell, 1974*b*), and it has been suggested that the effects of such drugs on intact cells may be mediated through effects on the intracellular Ca^{2+} concentration (see, e.g., Siddle & Hales, 1974).

Our results clearly indicate that in lymphocytes the effect of amphiphilic amines is to cause synthesis of phosphatidylinositol *de novo*, and therefore demonstrate that the effect of these drugs is different from the effect on phosphatidylinositol turnover produced by physiological stimuli which interact with cell-surface receptors. The results reported here give support to the hypothesis (Brindley & Bowley, 1975*a*,*b*; Eichberg & Hauser, 1974) that amphiphilic cationic drugs are inhibitors of phosphatidate phosphohydrolase.

Materials

The sources of many of the materials are recorded elsewhere: chlorpromazine, cinchocaine (Allan & Michell, 1974*a*), cocaine, procaine, fenfluramine, amphetamine (Brindley & Bowley, 1975*b*), deoxycholate (Lapetina & Michell, 1973*b*). Additional materials were trifluoperazine (Smith, Kline and French, Welwyn Garden City, Herts., U.K.), promethazine (May and Baker Ltd., Dagenham, Essex, U.K.), amethocaine (BDH, Poole, Dorset, U.K.), cetyltrimethylammonium bromide and Hepes buffer [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] (Hopkin and Williams, Chadwell Heath, Essex, U.K.), 2-phenethylamine and digitonin [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.]. Phytohaemagglutinin was obtained from Burroughs-Wellcome, Temple Hill, Dartford, Kent, U.K.

Methods

Cells were isolated from pig lymph nodes as described by Allan & Michell (1974*a*) and incubations were in a medium designated Hepes–Ringer: this medium, which contained 11mM-glucose, was similar to Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932) except that 18mM-Hepes buffer, adjusted to pH7.4 with NaOH, was substituted for HCO_3^-–CO_2.

Cells (2×10^8) were incubated in 1.0ml of Hepes–Ringer medium in test tubes at 37°C as follows. A solution of the compound whose effect was to be tested and either 10μCi of $^{32}P_i$ (10μCi/μmol), 0.5μCi [2-^{3}H]glycerol (420 or 5.0μCi/μmol) or 0.5μCi of [^{14}C]glycerol (5.2μCi/μmol) were put into tubes and the volume made up to 0.8ml with Hepes–Ringer medium. After 10min at 37°C the incubations were initiated by addition of 2×10^8 cells suspended in 0.2ml of Hepes–Ringer; these cell suspensions were warmed to 37°C before addition. After an appropriate period, which was usually 30min, incubations were terminated by addition of 3.75ml of methanol–chloroform (2:1, v/v), and lipids were extracted and separated by paper chromatography on formaldehyde-treated papers as described previously (Lapetina & Michell, 1972; Jones & Michell, 1974). The areas analysed from these papers were (*a*) that containing phosphatidylinositol, (*b*) a major spot containing most of the phospholipids of the cells, and (*c*) the area close to the solvent front in which the non-polar lipids migrated (see Plate 1*a* and Jones & Michell, 1974). When more detailed separations of different non-polar lipids or phospholipids were needed they were achieved by t.l.c. (Skipski *et al.*, 1964, 1968). Lipids were detected either on papers with Nile Blue or on either papers or t.l.c. plates with iodine vapour. Determinations of radioactivity were made by using papers which had been stained with iodine and from which the iodine had subsequently been allowed to evaporate. Appropriate areas of the chromatograms were cut out, and 10ml of water was added for ^{32}P determinations by Čerenkov counting or 10ml of scintillation fluid added for scintillation counting of ^3H or ^{14}C. Measurements were made in a Phillips liquid-scintillation counter and corrections for quenching were made by using appropriate channels-ratio methods.

Interactions between lipids and drugs were studied both by turbidimetric methods based on the observations of Feinstein (1964) and Blaustein (1967) and by investigating the effects of the drugs on the chromatographic behaviour of the lipids. In the former experimental system samples of the lipids were emulsified in 50mM-Tris–maleate buffer (pH7.0), and various quantities of cationic drugs were added. The resulting turbidity was measured spectrophotometrically at 350nm. In the latter experiments the drugs and lipids were mixed in the same manner and the complexes extracted by the normal procedure for lipid extraction. The chloroform solutions were chromatographed on formaldehyde-treated papers; a typical chromatogram is shown in Plate 1*c*. The lipids investigated were phosphatidylinositol prepared from bovine liver by the method of Lapetina & Michell (1973*b*), phosphatidylglycerol from *Micrococcus lysodeikticus* (kindly donated by Dr. V. Long) and phosphatidic acid prepared from egg phosphatidylcholine by phospholipase C treatment (kindly donated by Dr. R. Coleman.

Results

Effects of cinchocaine

In most of the experiments used to define the response of lymphocytes to amphiphilic cations the drug used was cinchocaine, a local anaesthetic, but the pattern of response to all the drugs was essentially similar. The results shown in Figs. 1 and 3 represent typical experiments with this drug: essentially similar data were obtained in one to four other experiments under each set of experimental conditions.

Addition of 0.4mM-cinchocaine to lymphocytes provoked a large increase (100–300-fold) in the rate of incorporation of $^{32}P_i$ into phosphatidylinositol (Fig. 1). This was accompanied by a much smaller increase in total labelling in the area of the chromatograms containing most of the other phospholipids of the cell (Fig. 1). Radioautography revealed that the latter effect was a composite one consisting of a decrease in labelling in the area occupied by phosphatidylcholine and phosphatidylethanolamine and an increase in labelling of two compounds running slightly ahead of the major lipids (Plates 1*a* and 1*b*); preliminary experiments suggest that these compounds might be phosphatidate and phosphatidylglycerol. The labelled component with a lower mobility than phosphatidylinositol is likely to be diphosphoinositide, whose labelling would rise as a secondary result of the rise in the specific radioactivity of phosphatidylinositol. The effect of cinchocaine appeared different from that of phytohaemagglutinin, in that the latter compound elevated labelling of phosphatidylinositol without any appreciable effects on the labelling of other lipids (Plates 1*a* and 1*b*).

Increased labelling of phosphatidylinositol was detected 2–5min after addition of cinchocaine and continued for several hours, with a steady decline in rate throughout the period of incubation (Fig. 1). This increased labelling was accompanied by a rise in the phosphatidylinositol content of treated cells. After 1h this value had risen by 25–50%, after 2h by about 50% and after 3.5h the phosphatidylinositol content had doubled (Figs. 2 and 3*a* and Table 1). No change was detected in the concentration of other phospholipids (Table 1).

The dose–response curve for the effect of cinchocaine on ^{32}P incorporation into phosphatidylinositol is shown in Fig. 3(*a*); it is typical of those obtained with a variety of amphiphilic amines. Fig. 3(*b*) shows that cinchocaine also greatly stimulated the labelling of phosphatidylinositol from [2-^{3}H]glycerol, which measures that fraction of glycerolipid synthesis that derives its phosphatidate precursor via acylation of glycerol 3-phosphate. Results obtained in experiments with [^{14}C]glycerol, which measure synthesis via both the glycerol phosphate and dihydroxyacetone phosphate pathways, were very similar except that the distribution of radioactivity in the non-polar lipid and major phospholipid fractions was somewhat shifted in favour of the phospholipids when compared with the results obtained with [2-^{3}H]glycerol (Fig. 3*c*). The simplest interpretation of these observations is that cinchocaine provoked synthesis *de novo* of the entire phosphatidylinositol molecule,

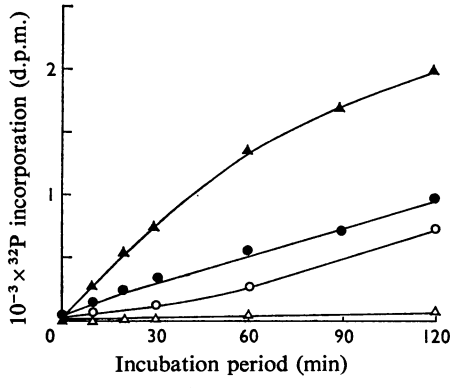


Fig. 1. *Effects of cinchocaine on the time-course of incorporation of ^{32}P into lymphocyte phospholipids*

Cells were incubated in the absence of cinchocaine (△, ○) or in the presence of 0.4mM-cinchocaine (▲, ●). Lipids were extracted, and radioactivity was measured in phosphatidylinositol (△, ▲) and in other phospholipids (○, ●). This experiment is a typical example selected from four essentially similar experiments.

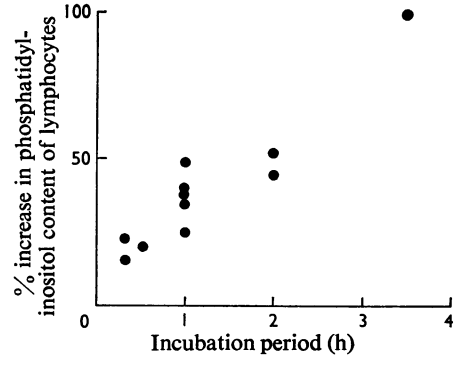


Fig. 2. *Increase in the phosphatidylinositol content of lymphocytes incubated with* 0.4mM-*cinchocaine*

The phosphatidylinositol contents of lymphocytes incubated in the absence and presence of cinchocaine were measured. The changes are expressed as percentages of the appropriate control value. Each point represents a different preparation of lymphocytes and is the mean of from two to four replicate determinations. Incubation conditions were as described in the Methods section.

Table 1. *Phospholipid composition of lymphocytes incubated with cinchocaine*

Cells were incubated with or without 0.4mM-cinchocaine for 3.5h at 37°C. Units are nmol of phospholipid/10^8 cells. The results are the means±S.E.M. of quadruplicate determinations.

	Unincubated control	3½h control	3½h+cinchocaine
Phosphatidylinositol	14±1	15±1.5	29±2
Phosphatidylserine	18±1.5	19±1.5	18±2
Major phospholipids	165±6	153±3	165±8

as had already been clearly indicated by the direct analyses of cellular phosphatidylinositol content.

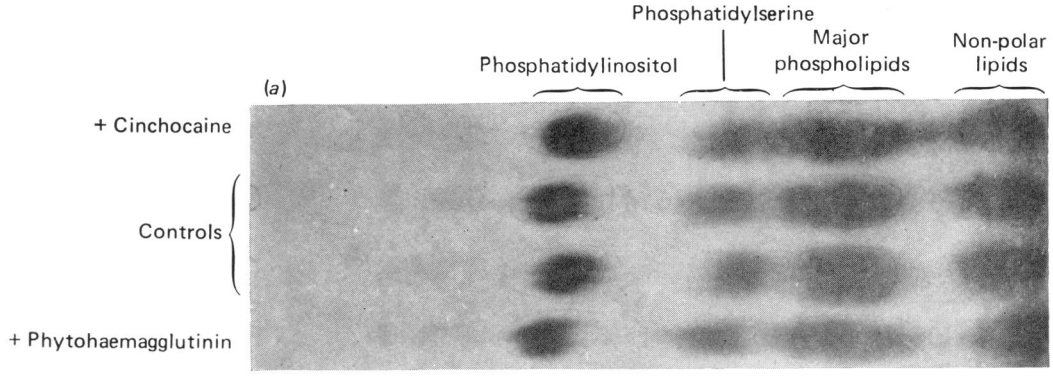
The dose–response curves for the effects of cinchocaine on labelling from [2-^{3}H]glycerol or [^{14}C]-glycerol of different groups of lipids showed marked differences between the behaviour of phosphatidylinositol and that of the other phospholipids and of the non-polar lipids (Figs. 3*b* and 3*c*). At low concentrations there was always a marked and parallel rise in labelling both of the major phospholipids and of the non-polar lipids. Confirmation of this was obtained by subfractionation of these groups by t.l.c. About 80% of the radioactivity of phospholipids labelled with [^{3}H]glycerol was in phosphatidylcholine and about 95% of that in non-polar lipids was associated with triacylglycerol. The distribution of labelled glycerol among the lipids was unchanged during the rise in their specific activities. It seems likely that this effect, which was also seen when [^{14}C]glycerol was the labelled precursor, was due to an increase in specific radioactivity of a glycerol-labelled precursor pool within the cells.

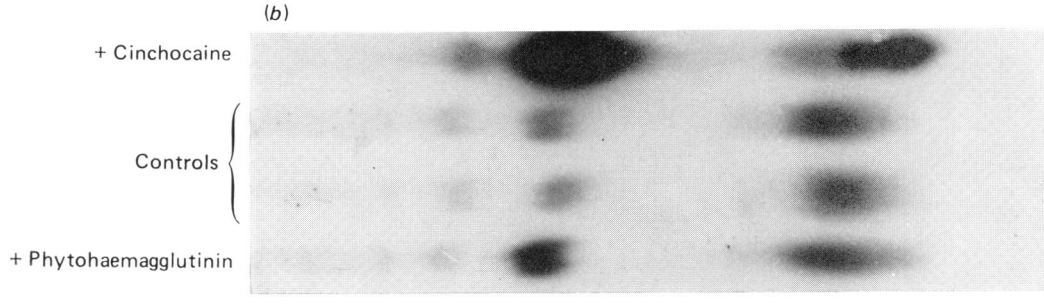
At concentrations of cinchocaine between 0.1 and 0.4mM the labelling of phosphatidylinositol with [^{3}H]glycerol or [^{14}C]glycerol increased rapidly and the labelling of the other two groups of lipids decreased (Figs. 3*b* and 3*c*). Above 0.4mM there was little further change in the radioactivity of any of these components. The fall in the radioactivity of lipids other than phosphatidylinositol appeared, at each cinchocaine concentration, to be about equivalent in magnitude to the rise in labelling of phosphatidylinositol. It therefore appeared that cinchocaine had little effect on the total synthesis of glycerolipids *de novo* by lymphocytes and that the major effect of the drug was to redirect about one-half to two-thirds of total glycerolipid synthesis into phosphatidylinositol and away from triacylglycerol and phosphatidylcholine. Although the changes in labelling of phosphatidylinositol at different cinchocaine concentrations were similar for the three labelled precursors, including $^{32}P_i$, there may appear to be a discrepancy in relation to the labelling of the other phospholipids. This is not, however, true since the higher cinchocaine concentrations decrease the labelling of the major phospholipids with ^{32}P (see above and Plate 1*b*). At very high drug concentrations labelling of all lipids, from any of the precursors, declined: this has been observed previously and is probably due to interference with ATP synthesis in cells exposed to high drug concentrations (Magee *et al.*, 1963; Brossard & Quastel, 1963).

The enhanced labelling of phosphatidylinositol from $^{32}P_i$ was unaffected by changes in the extracellular Ca^{2+} concentration between 10nM and 1mM. Added cytidine (5mM) or inositol (10mM) did not enhance labelling further, suggesting that in the first 30min of incubation these compounds did not become rate-limiting for phosphatidylinositol synthesis by these cells. Digitonin (0.1mM) or deoxycholate (1–100μM), which are respectively uncharged and anionic amphiphilic molecules, did not increase labelling of phosphatidylinositol; 0.1mM-deoxycholate was inhibitory, probably owing to cell lysis. Cyclic AMP and cyclic GMP (0.1mM) had no effects.

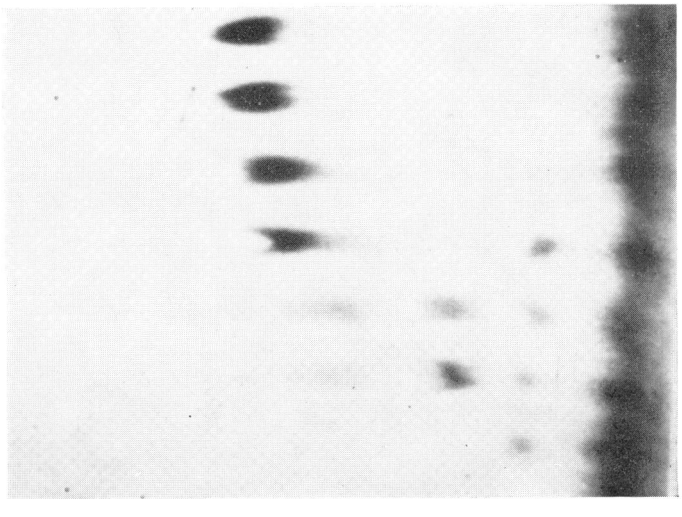
Effects of other cationic compounds

A variety of amphiphilic amines stimulated phosphatidylinositol synthesis (Table 2). Of these, phenothiazine derivatives (chlorpromazine, trifluoperazine and promethazine) were the most effective, producing half-maximum effects at 60–90μM. Although trifluoperazine was consistently the most effective there was relatively little difference in effectiveness between these compounds even though they vary markedly in their potencies as tranquillizers and local anaesthetics. Other amines with large hydrophobic substituents (e.g. imipramine and cinchocaine) were also very effective, whereas those with less bulky apolar substituents were relatively less effective (amphetamine, 2-phenethylamine, procaine). In some cases (chlorpromazine, cinchocaine, cetyltrimethylammonium) progressive increases in the concentration of a compound first raised and subsequently inhibited the labelling of phosphatidylinositol. The decrease sometimes occurred after maximal synthesis had been achieved (chlorpromazine, cinchocaine) but sometimes it was not possible to achieve maximal rates of synthesis before inhibition intervened (cetyltrimethylammonium, see Table 2). Microscopic inspection of the cells suggested that high drug concentrations which caused inhibition of labelling were producing cell lysis.





Phosphatidylinositol (nmol)	Cinchocaine (nmol)
40	–
40	100
40	200
40	400
40	1000
40	1500
–	400



EXPLANATION OF PLATE 1

Effects of cinchocaine ana phytohaemagglutinin on ^{32}P incorporation into lymphocyte phospholipids and the effect of cinchocaine on the chromatographic behaviour of phosphatidylinositol

Plates 1(*a*) and 1(*b*) show respectively a photograph of a chromatogram stained with Nile Blue and a radioautogram of the same chromatogram (3 days' exposure, Kodak BB 54 X-ray film). The lipids were extracted from cells which had been incubated with $^{32}P_i$ for 1 h in the absence of any additions (control), or with 0.4 mM-cinchocaine or 20 μg of phytohaemagglutinin per ml. Plate 1(*c*) shows a photograph of a chromatogram in which the effect of cinchocaine on the mobility of phosphatidylinositol is illustrated; 40 nmol of phosphatidylinositol and cinchocaine (0–1500 nmol, as specified) were mixed in 1 ml of water, and this mixture was subjected to lipid extraction and chromatography in the manner described for cells. The chromatogram was stained with Nile Blue. A large number of essentially similar observations were made with cinchocaine and also with other amphiphilic cationic drugs.

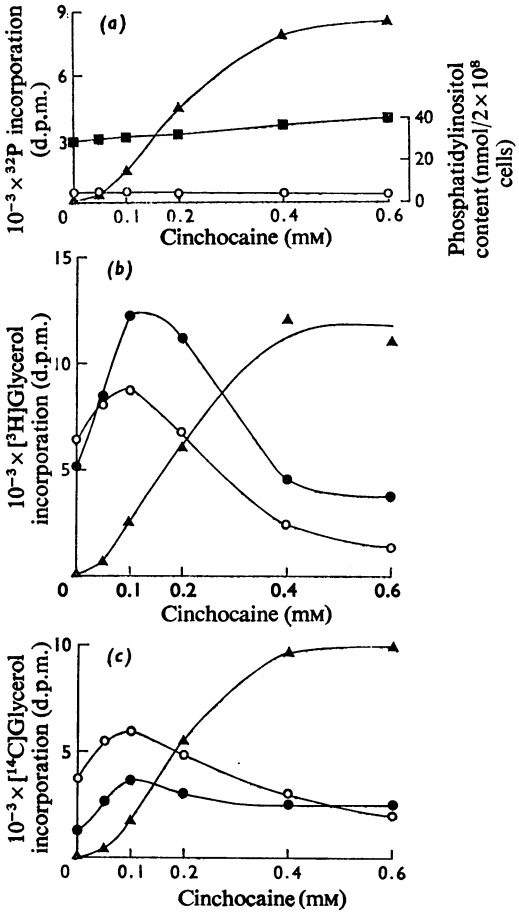


Fig. 3. *Effects of various concentrations of cinchocaine on the incorporation of* $^{32}P_i$, 2-[^3H]*glycerol and* [^{14}C]*glycerol into lymphocyte lipids and the increase in cellular phosphatidylinositol provoked by the drug*

Cells were incubated for 30min in the presence of the indicated concentration of cinchocaine and (*a*) $^{32}P_i$, (*b*) 2-[^{3}H]glycerol or (*c*) [^{14}C]glycerol. Radioactivity was measured in phosphatidylinositol (▲), the major phospholipids (○) and non-polar lipids (●). (*a*) also records the phosphatidylinositol content of the cells at different times during the incubations (■). The data with the three different radioactive precursors were obtained simultaneously on the same batch of lymphocytes. The data represent one of two experiments in which all three precursors were studied: essentially similar data were obtained in three other experiments in which only $^{32}P_i$ and 2-[^{3}H]glycerol were used.

Interactions between cationic drugs and anionic lipids

It seemed likely that the effects which were being observed occurred as a result of physical interactions between the cationic drugs and anionic phospholipids, particularly phosphatidate, in the treated cells. Preliminary experiments were therefore carried out to determine whether all of the active compounds showed such interactions. These could be detected either by measuring the ability of drugs to cause an increase in the particle size and turbidity of dispersions of phosphatidylinositol, phosphatidylglycerol and phosphatidate (Feinstein, 1964; Blaustein, 1967) or by determining whether drugs interfered with the chromatographic behaviour of anionic lipids on formaldehyde-treated papers. An example of the effects of cinchocaine on the mobility of phosphatidylinositol in the latter system is shown in Plate 1(*c*).

All the compounds which were active in enhancing phosphatidylinositol synthesis showed interactions with these lipids in one or both of these experimental procedures.

Table 2. *Effects of amphiphilic cationic compounds on phosphatidylinositol synthesis*

The values for trifluoperazine, chlorpromazine, cinchocaine and fenfluramine are means±S.E.M. (no. of experiments with different lymphocyte preparations). For other compounds either the mean values from two experiments which did not differ by more than 25% or an individual value are shown.

Compound	Concentration needed to elicit a response equal to 50% of the maximal response to cinchocaine (μM)
Trifluoperazine	68±10 (3)
Promethazine	80 (1)
Chlorpromazine	85±4 (3)
Imipramine	105 (2)
Cinchocaine	212±5 (5)
Cetyltrimethylammonium bromide	≈525* (2)
Amethocaine	485 (2)
Fenfluramine	717±160 (3)
Amphetamine	3750 (2)
2-Phenethylamine	5500 (2)
Procaine or cocaine	No effect up to 10mM (1)

* This value is extrapolated: cetyltrimethylammonium bromide was equal in potency to amethocaine at concentrations up to 0.3–0.5mM, but caused inhibition above this concentration.

Discussion

The range of tissues which show enhanced labelling of phosphatidylinositol when exposed to various amphiphilic cationic drugs has now been extended to include lymphocytes. It has also been demonstrated that this increase in labelling of phosphatidylinositol is due to synthesis *de novo* and that this synthesis occurs at the expense of an approximately equivalent decrease in the rates of synthesis of the other major

glycerolipids of the cell. The conclusion that extra phosphatidylinositol was synthesized *de novo* in treated cells arises from two sets of observations. First, these drugs caused increased incorporation of both glycerol and phosphate into phosphatidylinositol to approximately similar extents. Second, direct chemical analysis showed that the treated cells contained more phosphatidylinositol than the untreated cells. Treatment with a maximally effective dose of cinchocaine produced a rise in the cell content of phosphatidylinositol by about one-third in 1 h, and about one-half to two-thirds of the labelled glycerol incorporated into lipids went into phosphatidylinositol. In contrast, lymphocytes exposed to phytohaemagglutinin or other mitogenic stimuli exhibit (*a*) enhanced labelling of phosphatidylinositol from $^{32}P_i$ without a decrease in the labelling of other phospholipids (Fisher & Mueller, 1968, 1971; Masuzawa *et al.*, 1973; Maino *et al.*, 1975; and Plates 1*a* and 1*b*), and (*b*) a much smaller enhancement of phosphatidylinositol labelling when labelled glycerol is the precursor used (Fisher & Mueller, 1971; D. Allan & R. H. Michell, unpublished work). This response, like the enhanced phosphatidylinositol metabolism in a variety of other cells exposed to extracellular stimuli, is almost certainly an increase in the turnover of phosphatidylinositol rather than in its synthesis *de novo* (see Michell, 1975). Approx. 5nmol of phosphatidylinositol, and therefore about 8–10nmol of total glycerolipids, were synthesized in 1h in 10^8 cinchocaine-treated cells which contained about 200nmol total glycerophospholipids (Fig. 2, Table 1). This indicates that quiescent lymphocytes synthesize, and presumably degrade, about 5% of their glycerolipids in 1h. This calculation obviously rests on the assumptions that (*a*) the total rate of glycerolipid synthesis is not changed by these compounds and (*b*) the syntheses both of phosphatidylinositol and of the other glycerolipids draw on the same pools of glycerophosphate and phosphatidate; the information in this paper and the accompanying paper (Brindley & Bowley, 1975*b*) suggests that neither of these assumptions are likely to be grossly in error. Although approximate, this form of calculation allows a direct estimate to be made of the rate of glycerolipid synthesis *de novo* in a tissue; this value is normally rather difficult to obtain, but is potentially a useful item of information. Similar calculations should be possible with any system in which redirection of lipid synthesis by amphiphilic amines leads to a chemically detectable change in concentration of one of the affected lipids.

The most obvious way of shifting glycerolipid synthesis away from triacylglycerols, phosphatidylcholine and phosphatidylethanolamine and into phosphatidylinositol would be by inhibition of phosphatidate phosphohydrolase, which lies at the branch point at which synthesis of the major glycerolipids diverges from that of phosphatidylinositol and the other lipids which are synthesized via CDP-diacylglycerol. The experiments of Brindley & Bowley (1975*a*,*b*) have demonstrated directly that a variety of amphiphilic cationic drugs are effective inhibitors of this enzyme. Comparison of our measurements of rates of phosphatidylinositol synthesis in lymphocytes (Table 2) with their studies of the soluble phosphatidate phosphohydrolase of liver (Table 1 of Brindley & Bowley, 1975*b*) shows that the relative potencies of several drugs for modifying these two processes are very similar. The only marked difference between liver and lymphocytes seems to be that phosphatidate accumulates in liver treated with amphiphilic cations (Brindley & Bowley, 1975*a*,*b*) whereas it is largely converted into phosphatidylinositol in treated lymphocytes.

When the experiments of previous workers who have investigated the effects of cationic amphiphilic drugs on glycerolipid metabolism in various tissues (see the introduction) are examined in the knowledge that such drugs inhibit phosphatidate phosphohydrolase it appears that this effect alone could explain most, if not all, of the reported phenomena. In particular, it would lead to the observation that labelling of phosphatidate and/or phosphatidylinositol is usually stimulated by these compounds and also to the observation of Eichberg and his colleagues (Eichberg *et al.*, 1973*a*,*b*; Eichberg & Hauser, 1974) that there can be increased synthesis of all lipids derived from phosphatidate via the CDP-diacylglycerol pathway (i.e. CDP-diacylglycerol, phosphatidylglycerol, phosphatidylinositol, diphosphatidylglycerol). In lymphocytes only a small amount of labelling appeared in the material tentatively identified as phosphatidylglycerol, a mitochondrial lipid, whereas in pineal gland this was a major labelled product; this difference may reflect the relatively small number of mitochondria in lymphocytes.

The mechanism by which amphiphilic cations inhibit phosphatidate phosphohydrolase and stimulate phosphatidylinositol synthesis has not yet been elucidated. However, it seems very likely that these molecules interact directly with phosphatidate and neutralize its negative charge and that the neutralized phosphatidate is no longer recognized as a substrate by the enzyme. In support of this view, it is apparent that those cations with bulky hydrophobic groupings (e.g. phenothiazine and imipramine derivatives and cinchocaine) are the most effective agents in modifying glycerolipid synthesis, whereas the much less hydrophobic amphetamine and procaine were relatively ineffective. This overall pattern is very similar to that shown by the enzyme in lymphocytes which cleaves the glycerol-phosphate bond of another anionic phospholipid, phosphatidylinositol. This enzyme also has a pH optimum around 7.0, occurs in

the cytosol and shows a preference for anionic substrate; neutralization of the substrate by addition of cinchocaine, chlorpromazine or bivalent cations leads to its inhibition (Allan & Michell, 1974*a*,*b*). In contrast with this it should be noted that CTP–phosphatidate cytidylyltransferase, which catalyses synthesis of CDP-diacylglycerol from phosphatidate, must have been functioning satisfactorily even in those cells exposed to drug concentrations sufficient to markedly inhibit phosphatidate phosphohydrolase.

The suggestion that inhibition is due to the neutralization of the substrate of phosphatidate phosphohydrolase is supported by a calculation based on the amount of chlorpromazine needed to half-maximally stimulate phosphatidylinositol synthesis. This concentration (about 80μM) is roughly equal to the total concentration of anionic phospholipids in lymphocyte suspensions at 2×10^8 cells/ml (about 70μM-phosphatidylinositol plus phosphatidylserine). Since under these conditions about one-half of the chlorpromazine would be bound to membrane lipids (partition coefficient approx. 1000) it would appear that neutralization of about 50% of the cell's anionic phospholipids led to about 50% inhibition of phosphatidate phosphohydrolase.

Unfortunately the phenomenon which has most frequently led to the detection of effects of amphiphilic cations on lipid metabolism, i.e. enhanced labelling of phosphatidate and/or phosphatidylinositol with ^{32}P, appears superficially very similar to the enhanced phosphatidylinositol labelling which occurs in many cells as a response to interaction of appropriate agonists with cell surface receptors. It is clear from these experiments, however, that the two responses differ fundamentally in their mechanisms; the former is primarily an increase in the synthesis of phosphatidylinositol *de novo* whereas the latter involves a rapid turnover of only the phosphorylinositol group of the phosphatidylinositol molecule (see Michell, 1975). It is essential that any future studies of modifications to phosphatidylinositol metabolism should include experiments designed to differentiate between these two types of responses.

One of the most interesting, but unexplained, conclusions to emerge from the observations in the present paper and from our previous studies of phosphatidylinositol breakdown catalysed by a soluble fraction from lymphocytes (Allan & Michell, 1974*a*,*b*) is that pig lymphocytes have a remarkable capacity for both synthesis and breakdown of phosphatidylinositol. They appear potentially capable of breaking down their entire phosphatidylinositol complement in only a few seconds and of its synthesis in about 3h. It may be that these activities, which are not called on in the quiescent lymphocytes, supply the enzymic basis for the rapid increase in phosphatidylinositol turnover which occurs in activated lymphocytes; this has characteristics which indicate that it may be important to the mechanism whereby these cells become activated (Fisher & Mueller, 1968, 1971; Masuzawa *et al.*, 1973; Maino *et al.*, 1975).

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