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Chlorpromazine and Human Platelet Glycerolipid Metabolism: Precursor Specificity and Significance of Drug–Platelet Interaction Time

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ABSTRACT. Chlorpromazine is known to have a number of effects on glycerolipid metabolism in a variety of cell types, and in some cases reports are contradictory. To investigate the basis for some of these discrepancies, we reinvestigated the effects of chlorpromazine on some aspects of platelet glycerolipid metabolism. Time-courses conducted with [^{3}H]glycerol or [^{3}H]palmitic acid showed that the effects of chlorpromazine on the labelling of phosphatidylcholine, diacylglycerol, and triacylglycerol were highly dependent upon platelet–drug interaction time. The time-dependent changes in labelling patterns were independent of the presence of radiolabel during incubation, and were not the results of time-dependent changes in the platelets per se. The effects of chlorpromazine on the labelling of platelet glycerolipids by [^{3}H]glycerol, [^{3}H]palmitic acid, [^{32}P]P_i ([^{32}P]phosphatase), and [^{14}C]choline were compared. Dose–response curves conducted at 30-min incubation time showed that chlorpromazine potently inhibited labelling of diacylglycerol and diacyglycerol-derived lipids (triacyglycerol and phosphatidylcholine) by the ^{3}H-labelled precursors. Labelling of phosphatidylcholine by [^{32}P]P_i or [^{14}C]choline was, however, not affected at all by the drug. We conclude that the effects of chlorpromazine on platelets are highly time-dependent, and that the prolonged effects are most likely to be of biological significance. Furthermore, in platelets the effects of the drug on the labelling of phosphatidylcholine by isotope-labelled precursors are highly dependent on the route of incorporation of the specific precursor chosen. BIOCHEM PHARMACOL **57**;10:1113–1123, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. phenothiazine pharmacology; adrenergic beta agonist pharmacology; blood platelets; propranolol; chlorpromazine; glycerolipid metabolism

Chlorpromazine and other phenothiazines have been shown to have several effects on phospholipid metabolism, in particular to inhibit both the cytosolic and membrane-bound forms of the PAP† enzyme (EC 3.1.3.4) *in vitro* [1]. This effect has in turn been correlated to the ability of the same drugs to inhibit the incorporation of radiolabelled precursors into neutral phospholipids (PC and phosphatidylethanolamine) and into DAG and TAG [2, 3]. In addition, chlorpromazine can inhibit the synthesis of PC specifically through the inhibition of CTP:cholinephosphate cytidylyl transferase (EC 2.7.7.15) [4, 5], the key regulatory enzyme in PC biosynthesis.

In spite of these two possible inhibitory mechanisms with regard to the synthesis of PC, the net effect of chlorpromazine is variable. While in the studies cited above there was an inhibition of incorporation of radiolabelled precursors into PC, in other studies chlorpromazine failed to produce this effect. Rabkin and co-workers [6] found that chlorpromazine and trifluoperazine (10 μM) actually increased incorporation of [^{3}H]choline into PC in cultured chick heart cells preincubated for 24 hr with the drugs. Hoshi and co-workers [7] found that treatment of rats with 100 mg/kg chlorpromazine increased the mass of all phospholipids measured for 24 hr after administration. Maziére *et al.* [8] showed that trifluoperazine could increase fatty acid turnover in phospholipids in human fibroblasts in a time-dependent manner, with the maximal effect of trifluoperazine requiring 6-hr preincubation with the drug prior to the experiment. These results led us to believe that the choice of incubation time may be crucial to the results obtained when effects of phenothiazines on glycerolipid metabolism are studied.

Biosynthesis of PC is usually monitored as incorporation of isotope-labelled choline, inorganic phosphate, glycerol, or fatty acid. The latter three precursors all permit the coordinate measurement of all the glycerolipids. However, choline and phosphate are incorporated via CDP choline into PC through the CDP choline pathway (Fig. 1). Glycerol, on the other hand, is incorporated through the generation of DAG via PA, which represents the other branch of PC biosynthesis [9] (Fig. 1). In non-confluent cells or in liver cells, these pathways are believed to be

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† *Abbreviations:* DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidate; GFP, gel-filtered platelets; [^{32}P]P_i, [^{32}P]phosphate; and PAP, 3-*sn*-phosphatidate phosphohydrolase.

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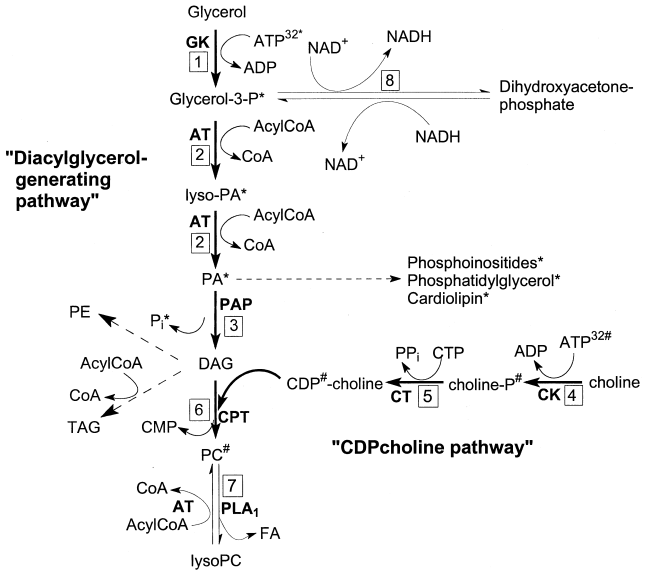

FIG. 1. Simplified scheme of glycerolipid biosynthesis from glycerol. GK, glycerol kinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30); AT, acyltransferase; AcylCoA, acyl coenzyme-A; PA, phosphatidate; PAP, 3-*sn*-phosphatidate phosphohydrolase (EC 3.1.3.4); DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; CT, CTP:choline-phosphate cytidylyl transferase (EC 2.7.7.15); CK, choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32); CPT, CDP choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2); PLA_1, phospholipase A_1 (phosphatidylcholine 1-acylhydrolase, EC 3.1.1.32) *, $[^{32}P]P_i$ incorporated in the glycerol kinase reaction. #, $[^{32}P]P_i$ incorporated in the choline kinase reaction.

coordinated in a one-to-one fashion to permit a tightly regulated synthesis of PC. However, in human platelets, the turnover of the glycerol moiety of PC as measured by the incorporation of [^{3}H]glycerol is considerably faster than the turnover of the phosphate moiety as measured by the incorporation of $[^{32}P]P_i$ [10]. Glycerol is incorporated into PC through the DAG pathway, while phosphate is incorporated through the CDP choline pathway (Fig. 1). It is therefore possible that the observed drug effect on PC metabolism depends upon which of the two pathways that is being monitored.

These considerations would have important implications for the design of studies regarding phenothiazine effects on glycerolipid metabolism and for the interpretation of data from such studies. We therefore investigated the effect of incubation time with chlorpromazine on the effects of the drug on labelling of various glycerolipids in human platelets by [^{3}H]glycerol and [^{3}H]palmitic acid. We then compared the effects of chlorpromazine on labelling of PC by [^{14}C]choline, $[^{32}P]P_i$, [^{3}H]glycerol, and [^{3}H]palmitic acid, respectively.

MATERIALS AND METHODS

Materials

$[^{32}P]P_i$ (PBS-11, carrier-free, 10 mCi/mL) and [^{14}C]choline (CFA424, 55 mCi/mmol, 200 μCi/mL) were from Amersham Life Science. [2-^{3}H]Glycerol (NET-022, 200 mCi/mmol, 1 mCi/mL) and [9,10-^{3}H(N)]palmitic acid (NET-043, 50 Ci/mmol, 5 mCi/mL) were from DuPont NEN (NEN Life Science Products). Chlorpromazine hydrochloride (>99%) was purchased from Norsk Medisinaldepot and propranolol (P-0884) from Sigma Chemical Co. Thin-layer sheets of alumina-backed silica (Silica gel 60) were from Merck. Lipid standards were from Doosan Serdary Research Laboratories.

Isolation of Platelets

Whole blood was obtained from healthy donors who attested to not having taken any drugs for 7 days prior to blood donation, from the Blood Bank at Haukeland Hospital, Bergen. The blood was anticoagulated with 1/6 volume of ACD (71 mM citric acid, 85 mM trisodium citrate, 111 mM glucose). Concentrated platelet-rich plasma was obtained by differential centrifugation as described previously [11]. Concentrated platelet-rich plasma was gel-filtered into Ca^{2+}-free Tyrode's buffer containing 5 mM glucose and 0.2% (w/v) BSA as described elsewhere [12]. Platelet numbers were determined after gel filtration with a ZM Coulter Counter (Coulter Electronics Ltd.) and adjusted to 3×10^8 platelets/mL. This suspension is referred to as gel-filtered platelets (GFP).

Incubations and Isotope Labelling

All the experiments were performed with only one radiolabelled precursor at a time.

PRELABELLING OF PLATELETS WITH $[^{32}P]P_i$. For studies with $[^{32}P]P_i$, the concentrated platelet-rich plasma was incubated for 60 min with 0.1 mCi/mL $[^{32}P]P_i$ at 37°. Excess isotope was removed by gel filtration. Phosphate was omitted from the Tyrode's buffer in order to avoid isotope dilution during the experiment. When the radioactivities of PI and PC were determined, samples were withdrawn from the GFP immediately before the experiments were started. The initial radioactivities of PI and PC were determined from these samples, and were subtracted from the radioactivites measured in the experiments. Thus, the ^{32}P radioactivites for PI and PC represent the net incorporation of $[^{32}P]P_i$ during the experiment. The comparability of the $[^{32}P]P_i$-prelabelling experiments with the other labelling experiments is explored further in the Discussion.

PHOSPHOLIPID BREAKDOWN ASSAY. For the phospholipid breakdown assay, concentrated platelet-rich plasma was incubated for 60 min with 15 μCi/mL of [^{3}H]glycerol and excess extracellular radioactivity removed by gel filtration as above. Prelabelled GFP were then prewarmed for 5 min and incubated for a further 2 hr in the presence or absence of 25 μM chlorpromazine, and duplicate samples were withdrawn at the indicated times.

DOSE–RESPONSE AND TIME-COURSE EXPERIMENTS. [^{32}P]P_i-labelling for these studies was as described above. Other isotopes were added after gel filtration, prior to the experiment. GFP was prewarmed for 5 min, and drugs (1:100 v/v) added at the start of the experiments. [^{3}H]palmitic acid (10 μCi/mL final concentration) was added to empty tubes prior to the experiment and the solvent evaporated before GFP was added. Thus, in this case the labelled precursor was present during prewarming. [^{3}H]glycerol (10 μCi/mL) and [^{14}C]choline (2 μCi/mL) was added at the start of the experiment, after prewarming of GFP. The platelets were then incubated for the given times, and samples were withdrawn. In the time-courses, aliquots were withdrawn from the same tube at the given times.

Extractions and Determination of Total and Lipid Radioactivity

TOTALS. For determination of total radioactivity of the platelets, 50 μL GFP was transferred into an ice-cold mixture of 125 μL formaldehyde(1M)/EDTA(50mM) + 450 μL 0.9% NaCl. The tubes were left on ice for at least 10 min to cool, and the platelets were pelleted by centrifugation at 1200 *g* for 2 min. The formaldehyde-fixed platelets were washed three times by resuspension in 500 μL buffer containing 0.9% NaCl/0.15M Tris, pH 7.4/EDTA(100 mM) (12:3:1), followed by centrifugation. The platelet pellets were finally resuspended in 0.1% Triton X-100, frozen and radioactivity determined by scintillation counting after thawing.

LIPIDS. For determination of radioactivity in lipids, duplicate samples (0.5 or 1.0 mL) were withdrawn directly into 4 volumes of ice-cold chloroform/methanol/concentrated HCl (20:40:1) and extracted as described by Bligh and Dyer [13]. With samples labelled by [^{3}H]glycerol, the lower chloroform phase was washed twice with 700 μL of an ice-cold solution of methanol/water/concentrated HCl (50:50:1) if PI was to be measured. The purpose of this procedure was to selectively remove contaminating radioactivity which had a retardation factor value slightly lower than PI in the thin-layer chromatographic system used.

Separation and Detection of Radiolabelled Lipids

Dried lipid extracts (solvents removed by an Ar jet) were dissolved in 40 μL chloroform and applied to aluminium-backed silica gel thin-layer sheets. Lipids were separated by thin-layer chromatography in a system containing chloroform/methanol/methylamine (40%)/water (60:36:5:5, by volume) for isolation of PC and PI, and in a system containing chloroform/acetic acid(glacial)/methanol/water (81:45:10:4) for isolation of PA. Another system containing ethylacetate/isooctane/glacial acetic acid/water (90:50:100:20, only the upper phase was used) was used for isolation of TAG and [^{3}H]glycerol-labelled DAG. Another system of petroleumether/ethylacetate (60:40) was used for the isolation of DAG labelled with [^{3}H]palmitic acid to avoid occasional overlap with free fatty acid in the former system. ^{32}P- or ^{14}C-labelled lipids were visualised and counted by a Packard InstantImager 2024, scraped off the TLC plates and the total radioactivity determined by scintillation counting. ^{3}H Radioactivity was either determined directly on the TLC sheets by a Raytest IM 3000 Radio-TLC Analyzer (RITA), or visualised by co-chromatography with standards and staining in iodine vapour. The stained spots were then scraped off the TLC sheets and radioactivity determined by scintillation counting.

Statistical Analysis

All data represent determinations done with blood from 3–6 different donors, and all determinations were performed in duplicate for each donor. Data from the dose–response experiments (Figs. 5 and 6) and from the preincubation experiments (Fig. 4) were analysed by the Bonferroni multiple comparison method. Data were considered significantly different from the corresponding controls when $P \leq 0.05$. Statistical analysis versus control values within a single curve were performed using the original dpm data or using data normalised by dividing by total radioactivity or total lipid radioactivity in controls (Fig. 6). Statistical analysis of differences between different curves for the same concentration of drug in Fig. 5 was performed on % of control values in order to be able to compare data for different isotope-labelled precursors. For the time-courses (Figs. 2 and 3), the data (dpm values) from chlorpromazine-treated platelets were compared to the corresponding controls by an unpaired Student's *t*-test, and were considered significant when $P \leq 0.05$.

RESULTS

Effect of Drug–Platelet Interaction Time

The effects of chlorpromazine (25 μM) on the labelling of platelet DAG, TAG, and PC by [^{3}H]glycerol and [^{3}H]palmitic over a time-span of 2 hr are shown in Fig. 2. At 30 min, there was a 90% inhibition of labelling of DAG by [^{3}H]glycerol, a complete block of labelling of TAG, and an 88% inhibition of labelling of PC by [^{3}H]glycerol. Similar results were observed with [^{3}H]palmitic acid, although inhibition of labelling of TAG at 30 min was not quite significant ($P = 0.055$). This was in great contrast to what was observed after 2 hr, when labelling of DAG and TAG by [^{3}H]glycerol increased by a factor of 18 (170 ± 44 to 3085 ± 1469 dpm/10^8 cells) and 2.5 (1071 ± 257 to 2665 ± 1260 dpm/10^8 cells), respectively, while labelling by [^{3}H]palmitic acid increased by a factor of 18 (DAG, 1141 ± 983 to 20674 ± 10799 dpm/10^8 cells) and 2 (TAG, 11366 ± 6083 to 23005 ± 8292 dpm/10^8 cells), respectively. Chlorpromazine did not stimulate the labelling of PC by either precursor at any time, but at 2 hr, the initial inhibitory effect of chlorpromazine on the labelling of PC was lost. The labelling of TAG and PC controls by both

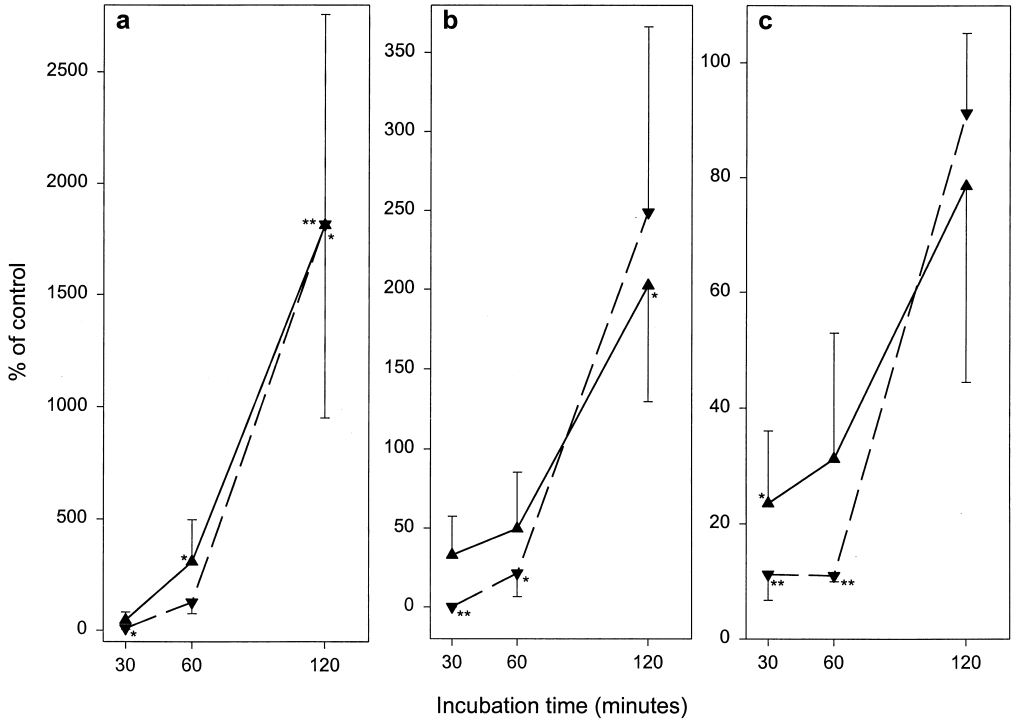

FIG. 2. Time-courses of the effect of chlorpromazine on the labelling platelet DAG (a), TAG (b), and PC (c) by [^{3}H]glycerol (▼) or [^{3}H]palmitic acid (▲). Gel-filtered platelets were incubated for the indicated times with the radioactive precursors (10 μCi/mL) in the presence of 25 μM chlorpromazine or the corresponding volume of 0.9% NaCl as described in the Materials and Methods section. Chlorpromazine-treated samples (3–5 individual experiments) were compared against controls (dpm data) by an unpaired student's *t*-test. Statistically significant data are labelled *($P \leq 0.05$), **($P \leq 0.01$), and *($P \leq 0.001$), respectively. Radioactive content of 30-min controls labelled with [^{3}H]glycerol were 193.1 ± 79.7 (DAG), 108.2 ± 34.1 (TAG), and 578.1 ± 179.9 (PC) dpm/10^8 cells. The corresponding values for the [^{3}H]palmitic acid-labelled controls were 4709.6 ± 2805.7 (DAG), 5310.8 ± 2603.7 (TAG), and 27212.7 ± 15660.1 (PC) dpm/10^8 cells.**

precursors increased in a nearly linear manner during the experiments (dpm data for Fig. 2). The control ^{3}H radioactivity of DAG was, however, nearly constant (between 170 and 193 dpm/10^8 cells) in the case of [^{3}H]glycerol, and decreased from 3139.8 ± 1870.5 to 1141.5 ± 983.3 in the case of [^{3}H]palmitic acid.

Total ^{3}H radioactivity within the platelets was not significantly affected by chlorpromazine during the experiments (data not shown), and the increase in labelling of lipids described above was therefore not caused by an increased uptake of precursors into the platelets over time.

The observed results could also be due to redistribution of radioactivity between the different lipids induced by chlorpromazine at the longer incubation times, e.g. by the activation of one or several phospholipases by the drug. This possibility was examined by performing an experiment similar to that in Fig. 2, using platelets prelabelled with [^{3}H]glycerol prior to gel filtration as described in the Materials and Methods section. The time-dependent changes in ^{3}H radioactivity of TAG, PI, PC, PA, and DAG are shown in Fig. 3. Control radioactivities were fairly constant over the 2-hr time-span except for TAG radioactivity, which decreased somewhat with time. There were no significant effects of chlorpromazine on the ^{3}H radioactivities of TAG, PI, or PC (Fig. 3a–c), which are all potential substrates for lipases or phospholipases. In the time-course (Fig. 2), chlorpromazine caused an increase in the radioactivity of DAG to values similar to that in PC (DAG = 3085 ± 1469 dpm, PC = 3346 ± 513 dpm) after 2 hr. The labelling of PC in control platelets in the prelabelling experiments was of a similar magnitude (4179 ± 1502 dpm). The data on PA (Fig. 3d) and DAG (Fig. 3e) were statistically inconclusive, because there were large percental variations in measurements from experiment to experiment due to the low (50–200 dpm) radioactive content of the lipids. The overall radioactivity of PA and DAG from chlorpromazine-treated platelets were, however, practically identical to control values. Furthermore, the largest increases in radioactivity measured in any single experiment were 64 dpm (DAG) and 46 dpm (PA), respectively, values far too small to account for the huge increases in radioactive content of e.g. DAG measured in the time-course experiments.

The time dependence of chlorpromazine's effects on the incorporation of [^{3}H]glycerol and [^{3}H]palmitic acid into platelet lipids could be due to changes within the GFP during incubation. This is particularly relevant in the current model system, since the responsivity of GFP to agonists is well known to decrease with time after gel filtration. Alternatively, it could be due to the effect of a time-dependent distribution of the isotopes within the cells, or related to the distribution of chlorpromazine itself.

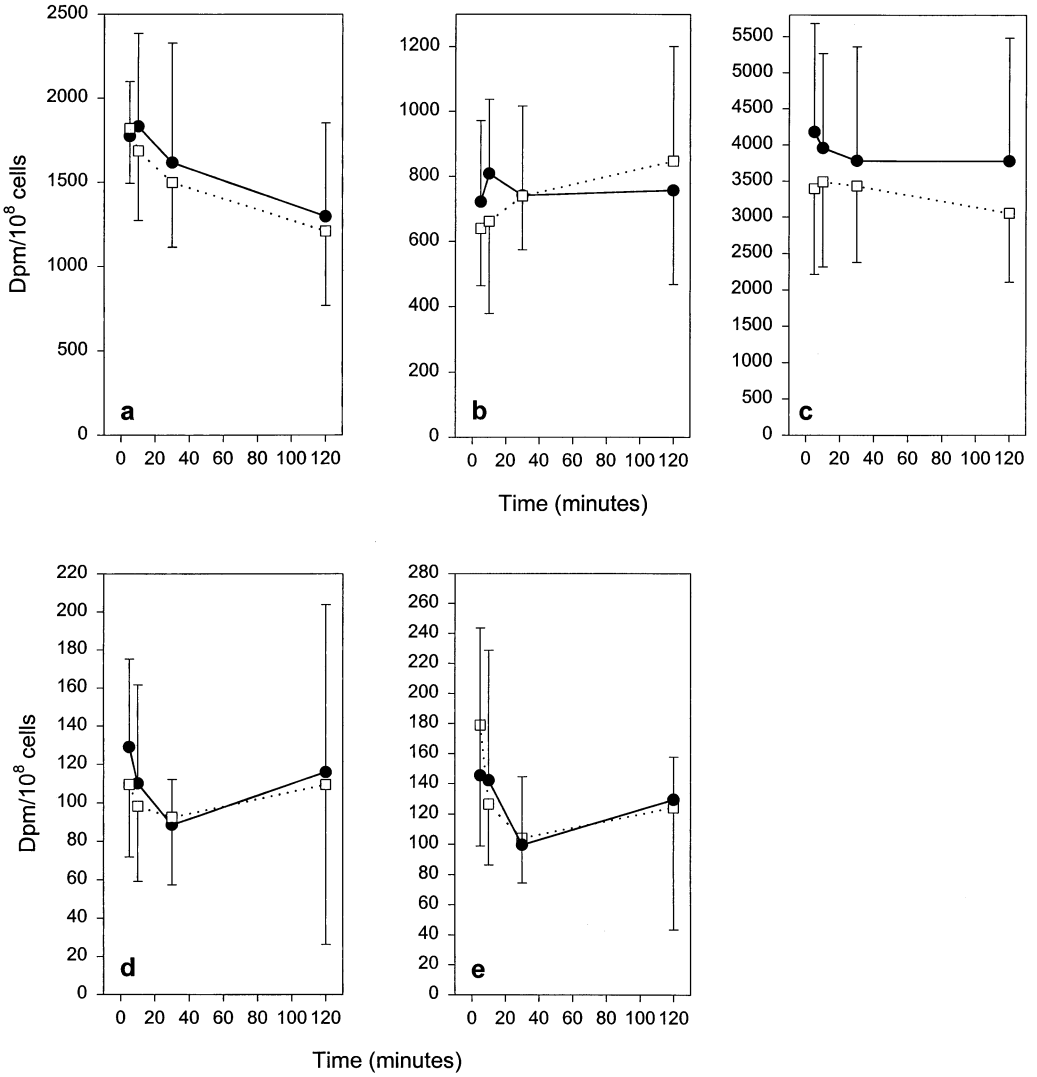

FIG. 3. Effect of chlorpromazine on ^3H radioactivity of platelet triacylglycerol (a), phosphatidylinositol (b), phosphatidylcholine (c), phosphatidic acid (d), and diacylglycerol (e) in platelets prelabelled with [^3H]glycerol. Platelet-rich plasma was incubated for 60 min in the presence of [^3H]glycerol (15 μCi/mL), and excess isotope was removed by gel filtration. Gel-filtered platelets were then incubated further for the indicated times in the presence of 25 μM chlorpromazine (open symbols) or the corresponding volumes of 0.9% NaCl (filled symbols). Data represent means ± SD of four different blood donors.

To distinguish between these two possibilities, we incubated platelets with [^3H]glycerol for a constant period of time (30 min) after the platelets had been preincubated for various times in the presence or absence of chlorpromazine. The results of these experiments, represented as percent changes in chlorpromazine-treated samples relative to the corresponding controls, are shown in Fig. 4. It is evident from these data that preincubation with chlorpromazine in the absence of [^3H]glycerol could produce changes in the effect of chlorpromazine qualitatively similar to those seen after 1 and 2 hr in the time-course studies (Fig. 2), although the time-dependent changes were quantitatively smaller in the experiment shown in Fig. 4. A 2-hr preincubation with 25 μM chlorpromazine did, for example, produce a 10.6-fold (106 ± 37 to 1121 ± 519 dpm) increase in labelling of DAG by [^3H]glycerol, while the corresponding value for the 2-hr time-course in Fig. 2 was 18-fold. Preincubation with NaCl did not affect the interaction of chlorpromazine with incorporation of [^3H]glycerol into platelet lipids. When the dpm values were compared by the Bonferroni multiple comparison method, the samples preincubated with 0.9% NaCl before treatment with chlorpromazine and [^3H]glycerol, were always significantly different ($P \leq 0.05$) from the corresponding samples preincubated in the presence of chlorpromazine (25 μM) for DAG and TAG. The same was the case for 10 μM chlorpromazine for PC.

Effects of Chlorpromazine on the Labelling of PC by Radiolabelled Precursors Incorporated through Different Pathways

The effect of chlorpromazine and propranolol on the incorporation of [^3H]glycerol, [^3H]palmitic acid, [^{32}P]P_i, and [^{14}C]choline into PC over a 30-min period is shown in

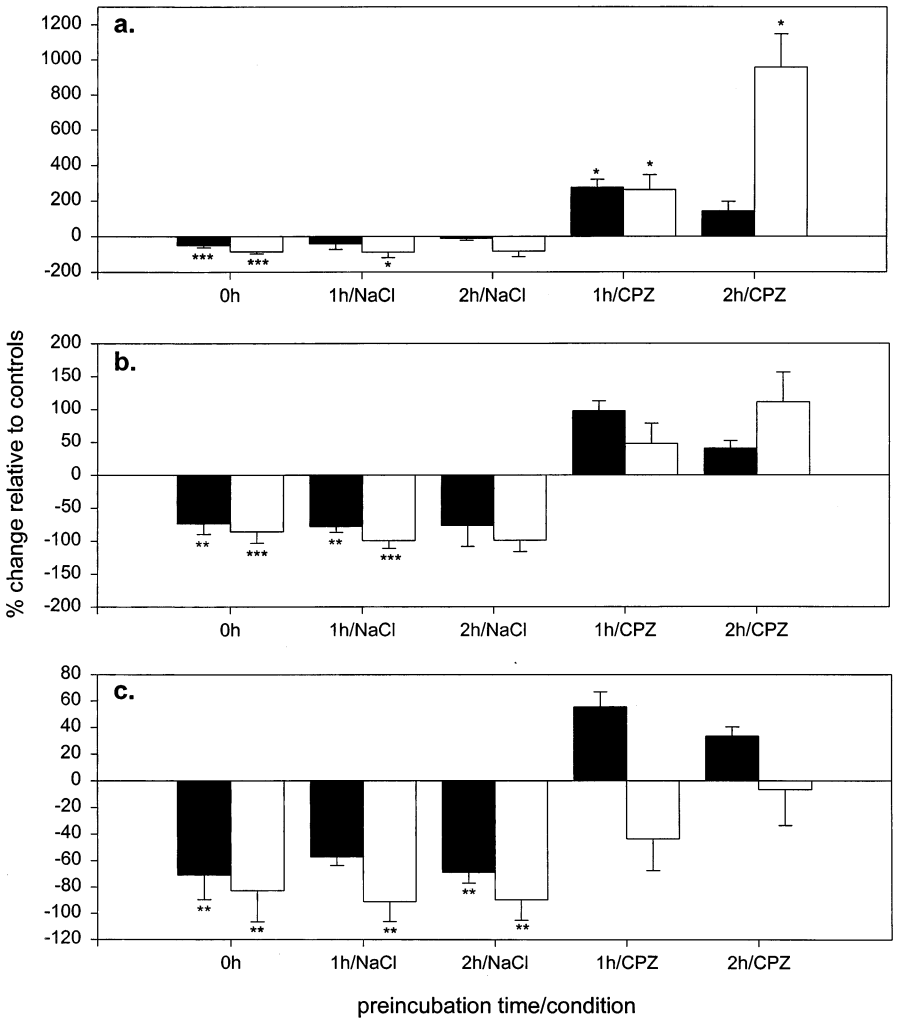

FIG. 4. Effect of chlorpromazine–platelet interaction time on the final effect of chlorpromazine treatment of platelets on the incorporation of [^{3}H]glycerol into platelet DAG (a), TAG (b), and PC (c). Gel-filtered platelets were preincubated for the time periods indicated in the presence of 10 or 25 μM chlorpromazine or the corresponding volume of 0.9% NaCl. After this preincubation, [^{3}H]glycerol (10 μCi/mL) was added to the platelet suspension, and incubation was continued for another 30 min in the presence of chlorpromazine (10 μM, filled bars or 25 μM, open bars) or the corresponding volume of 0.9% NaCl (controls). Data are represented as % difference in chlorpromazine-treated platelets relative to the corresponding controls. Data represent means ± SD from 3–6 different blood donors. The data were analysed by the Bonferroni multiple comparison method, and data significantly different from the corresponding controls are labelled *($P \leq 0.05$), **($P \leq 0.01$), and *($P \leq 0.001$), respectively. Control cpm-values were similar to the 30-min controls in Fig. 2.**

Fig. 5. The two first-mentioned precursors are incorporated into PC mainly through DAG (Fig. 1), although [^{3}H]palmitic acid can also be incorporated through fatty acid remodelling. The latter pair of precursors are incorporated through the CDP-choline pathway (Fig. 1). Propranolol was used as a positive control for PAP inhibition [1]. With [^{32}P]P_i as the precursor, prelabelled platelets were used as described in the Materials and Methods section, and no added phosphate was present during incubation with the drugs. The three other precursors were added at the beginning of the experiments, and incorporation of the precursors occurred in the presence of drugs. The comparability of the different experiments is considered in the Discussion. Chlorpromazine significantly inhibited incorporation of [^{3}H]glycerol by 87 ± 13% and [^{3}H]palmitic by 74 ± 12% (Fig. 5a). The dose–response relationships were similar for both precursors. The incorporation of [^{14}C]choline and [^{32}P]P_i was not affected at all by the drugs. Similar results were obtained with propranolol (Fig. 5b), although propranolol produced a slight increase in incorporation of [^{14}C]choline. Propranolol (20 and 30 μM) did produce a significant ($P \leq 0.05$, Bonferroni) 60% increase in the total [^{3}H]glycerol radioactivity content of the platelets (data not shown). Apart from this, the drugs had no significant effects on the uptake of any of the isotope-labelled precursors into the platelets.

The effects of the same drugs on the labelling of PI by [^{3}H]glycerol, [^{3}H]palmitic acid, and [^{32}P]P_i are shown in the insets of Fig. 5. Both drugs stimulated the labelling of PI by all three precursors to a similar degree in a dose-dependent manner, although the data on labelling by [^{3}H]palmitic acid were not statistically significant.

As mentioned above, [^{3}H]palmitic acid can be incorporated into PC by two separate pathways. There are also some concerns regarding the use of [2-^{3}H]glycerol as a precursor in these experiments (see Discussion). To circumvent these problems, two different precursors that are incorporated through separate enzyme reactions were used to monitor each pathway. Any effects of the drugs not attributable to a common pathway should result in divergence in the behaviour of the two precursors. A multiple, statistical comparison between the % of control values from Fig. 5 for the four different radiolabelled precursors at each concentration of drug is shown in Table 1. [^{3}H]Glycerol and [^{3}H]palmitic acid were never significantly differently affected by the same concentrations of drugs, as was the

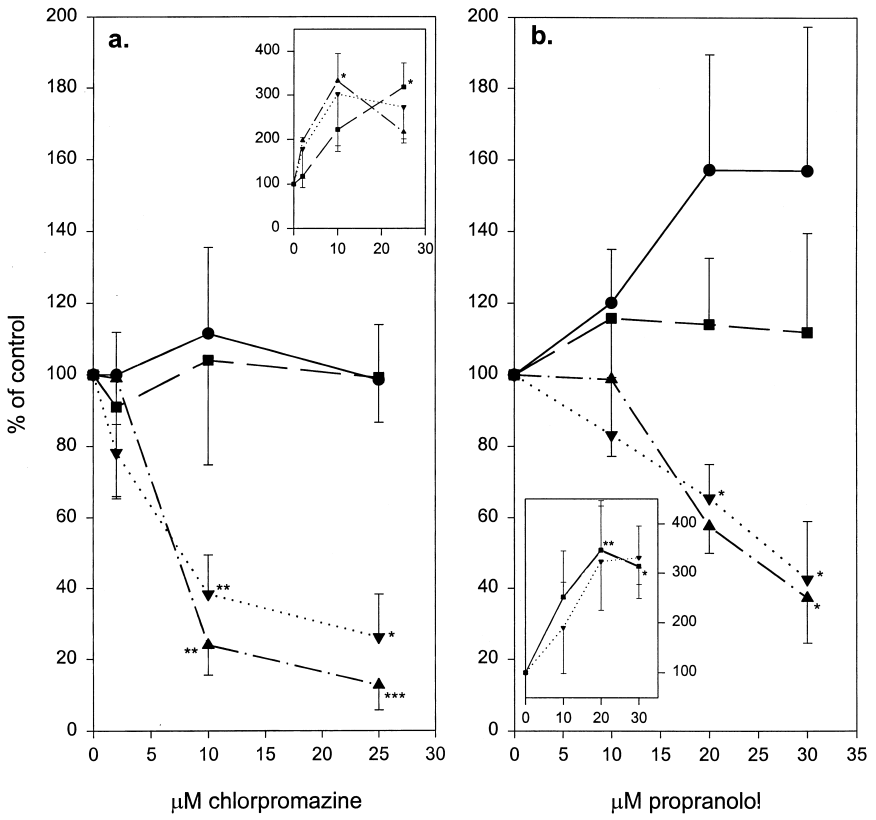

FIG. 5. Effects of chlorpromazine (a) and propranolol (b) on the labelling of platelet phosphatidylcholine by [^{32}P]P_i (■), [^{14}C]choline (●), [^{3}H]palmitic acid (▼), and [^{3}H]glycerol (▲). The effects of the drugs on the labelling of phosphatidylinositol by the latter three precursors are shown in the insets. Gel-filtered platelets were incubated for 30 min with the given concentration of drugs together with isotope-labelled precursors as described in the Materials and Methods section. With [^{32}P]P_i, prelabelled platelets were used. The initial radioactivities as measured immediately before the experiments were started were subtracted. The original dpm data were analysed by the Bonferroni method, and data points significantly different from the corresponding controls are labelled *($P \leq 0.05$), **($P \leq 0.01$), and *($P \leq 0.001$), respectively. In the inset of Fig. 5b, the significance labels apply to both the ^{32}P labelling and [^{3}H]glycerol labelling curves, which are not separable in the figure. Control values in the chlorpromazine experiments were: [^{14}C]choline, 302.9 ± 133.3; [^{32}P]P_i, 127.1 ± 4.4; [^{3}H]glycerol, 554.4 ± 154.4 dpm/10^8 platelets; and for [^{3}H]palmitic acid, 1951.1 ± 371.3 cpm/10^8 platelets. Control values in the propranolol experiments were: [^{14}C]choline, 486.1 ± 359.7; [^{32}P]P_i, 152.2 ± 64.7; [^{3}H]glycerol, 820.7 ± 368.1; and [^{3}H]palmitic acid, 2952.2 ± 612.1 dpm/10^8 platelets.**

case for [^{14}C]choline and [^{32}P]P_i. Furthermore, the two pathways were clearly affected by both drugs in a qualitatively different manner, since there were significant differences between the former and latter pairs of precursors for the highest concentrations of both drugs (Table 1).

Over a time-course of 2 hr, chlorpromazine (25 μM) did not significantly affect the incorporation of [^{32}P]P_i into PC (data not shown). If anything, there was a tendency towards increasing inhibition with time, but this effect was not significant.

It is evident from Fig. 2 that chlorpromazine potently inhibits labelling of DAG by either [^{3}H]glycerol or [^{3}H]palmitic acid at 30 min. The steady-state level of DAG may, however, be influenced by a number of factors. A true inhibition should therefore be measured as the total amount of precursor incorporated into lipids through DAG. Although we did not measure the labelling of phophatidylethanolamine in these experiments, the pooled data for DAG, TAG, and PC should provide a reliable estimate of the amount of [^{3}H]glycerol incorporated into platelet lipids through DAG. The pooled [^{3}H]glycerol data are shown in Table 2. Chlorpromazine (25 μM) significantly inhibited the total [^{3}H]glycerol incorporation by 91% at 30 min. Propranolol similarly (30 μM) inhibited incorporation by 58%, although in this case inhibition was not quite significant. Also included are the pooled data for DAG, TAG, and PC from the 60- and 120-min time points from Fig. 2. Although labelling of PC by [^{3}H]glycerol was not stimu-

TABLE 1. Statistical analysis of the data in Fig. 3

Test	PRO-10 μM	PRO-20 μM	PRO-30 μM	CPZ-2 μM	CPZ-10 μM	CPZ-25 μM
[^{3}H]Pal vs. [^{3}H]Gly	NS	NS	NS	NS	NS	NS
[^{3}H]Pal vs. [^{32}P]P_i	NS	$P \leq 0.05$	$P \leq 0.05$	NS	$P \leq 0.01$	$P \leq 0.001$
[^{3}H]Pal vs. [^{14}C]Cho	$P \leq 0.05$	$P \leq 0.001$	$P \leq 0.001$	NS	$P \leq 0.01$	$P \leq 0.001$
[^{3}H]Gly vs. [^{32}P]P_i	NS	$P \leq 0.05$	$P \leq 0.05$	NS	$P \leq 0.01$	$P \leq 0.001$
[^{3}H]Gly vs. [^{14}C]Cho	NS	$P \leq 0.001$	$P \leq 0.001$	NS	$P \leq 0.001$	$P \leq 0.001$
[^{32}P]P_i vs. [^{14}C]Cho	NS	NS	NS	NS	NS	NS

A statistical analysis of the difference in the effect of propranolol and chlorpromazine on the incorporation of various isotope-labelled precursors into phosphatidylcholine (Fig. 3) was conducted as follows. For each concentration of drug, the % of control values (N = 3–4) for each precursor were compared with the same data for all the other precursors using the Bonferroni multiple comparison method. The resulting significance levels are presented in the table. [^{3}H]Pal, [^{3}H]palmitic acid; [^{3}H]Gly, [^{3}H]glycerol; [^{14}C]Cho, [^{14}C]choline; [^{32}P]P_i, [^{32}P]phosphate. NS, not significant.

TABLE 2. Effects of chlorpromazine (CPZ) and propranolol (PRO) on the total incorporation of [^{3}H]glycerol into diacylglycerol + triacylglycerol + phosphatidylcholine

	dpm/10^8 cells				dpm/10^8 cells
μM CPZ	30 min	60 min	120 min	μM PRO	30 min
0	879.6 ± 287.1	1910.5 ± 579.5	4909.3 ± 994.2	0	1040.4 ± 446.8
2	874.6 ± 240.4			5	692.0 ± 568.2
10	221.2 ± 8.6*			10	990.3 ± 321.5
25	80.6 ± 33.9**	444.5 ± 153.0*	9095.6 ± 2475.8*	20	661.3 ± 179.5
				30	432.0 ± 80.5

Phosphatidylcholine radioactivities from the [^{3}H]glycerol labelling experiment in Fig. 5 were combined with the corresponding radioactivities for diacylglycerol and triacylglycerol. For chlorpromazine, the same data for the 60- and 120-min time-points from Fig. 2 are included. Dose–response data at 30 min were tested by the Bonferroni multiple comparison test as in Fig. 5. The time-course data (60 and 120 min) were tested by an unpaired student's *t*-test as in Fig. 2. Significance levels are indicated as *($P \leq 0.05$) and **($P \leq 0.01$), respectively.

lated by chlorpromazine at 2 hr, 25 μM chlorpromazine did significantly increase the total labelling of DAG, TAG, and PC by 85%.

To check whether inhibition of PAP could be a possible explanation for the inhibitory effects of chlorpromazine and propranolol at 30 min, we measured the effects of the drugs on the labelling of PA by [^{32}P]P_i. As shown in Fig. 6, both drugs stimulated the labelling of PA in a dose-dependent manner. When the ^{32}P radioactivities of PA controls were monitored for 2 hr, values remained low (90–160 dpm/10^8 cells) and did not increase with time (data not shown).

DISCUSSION

Basis for Interpretation of Radioisotope Labelling Data

In our previous experiments with [^{32}P]P_i, platelets were prelabelled and excess radiolabelled precursor was removed by gel filtration. Such GFP were suspended in phosphate-free Tyrode's solution to avoid dilution of isotope labelling by phosphate uptake. Immediately after gel filtration, the specific radioactivity of PI was 30–40 times lower than the specific activity of γ-phosphate of ATP, and both the absolute and specific radioactivities of PI continued to increase for at least 90 min [14]. One therefore has a pre-steady-state condition with regard to the labelling of PI by [^{32}P]P_i for at least 90 min. An increase in the total radioactivity of PI under these conditions therefore reflects the synthesis of new PI rather than an increase in its mass. The same conclusion can be drawn with regard to the labelling of PC by [^{32}P]P_i, since the mass of PC has been shown to be unchanged for 2 hr under the same conditions [10] and labelling of PC by [^{32}P]P_i increased nearly linearly in the absence of drugs in our experiments (original dpm values for Fig. 2c). In the present experiments, we further assured the comparability of the ^{32}P radioactivities to those obtained with the other radiolabelled precursors by measuring the initial ^{32}P radioactivities of PI and PC immediately before the experiments were started. These values were subtracted from the dpm values measured during incubation. Thus, ^{32}P data for PI and PC represent net incorporation of [^{32}P]P_i during the experiments.

All the other precursors used ([^{3}H]glycerol, [^{3}H]palmitic acid, and [^{14}C]choline) were added at the start of the experiments, allowing the platelets to incorporate radioprecursors during the experiment. Since the labelling of PI,

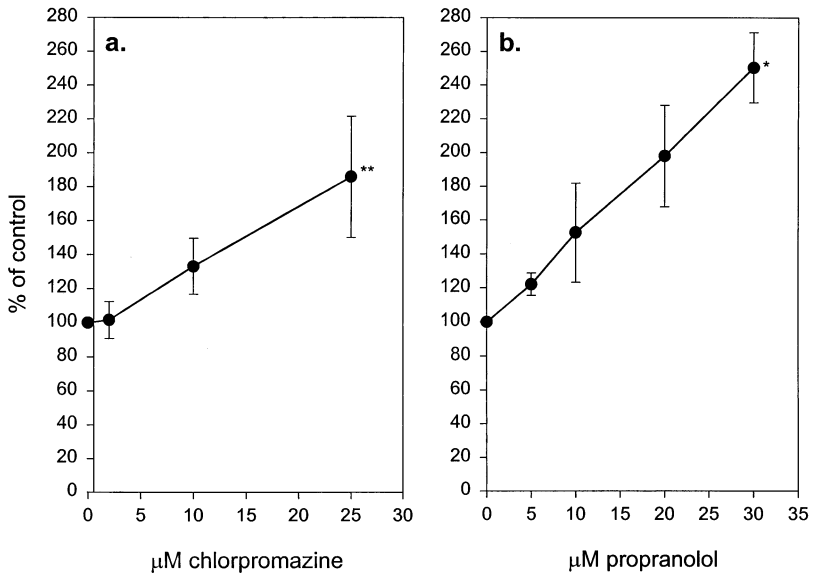

FIG. 6. Effect of chlorpromazine (a) and propranolol (b) on the incorporation of [^{32}P]P_i into phosphatidate. Gel-filtered platelets prelabelled with [^{32}P]P_i were incubated for 30 min in a phosphate-free buffer in the presence of the given concentrations of drug or the corresponding volume of 0.9% NaCl (controls). Data represent means of 4 different donors ± SD of ^{32}P content of phosphatidate. Statistical analysis was performed by the Bonferroni method on data normalised by dividing by total phospholipid ^{32}P content in NaCl controls. Values significantly different from controls are labelled *($P \leq 0.05$) and **($P \leq 0.01$), respectively.

PC, and TAG by these precursors increased nearly linearly throughout the experiments in the absence of drugs (original dpm data for Fig. 2), steady-state conditions were not reached during the experiments. We therefore conclude that in our experiments, labelling of PI, PC, or TAG by either of the radiolabelled precursors used occurred at pre-steady-state conditions. DAG and PA are both metabolic intermediates in glycerolipid biosynthesis (Fig. 1). Labelling of these intermediates by [^{3}H]glycerol/palmitic acid (DAG) or [^{32}P]P_i (PA) did not occur at pre-steady-state conditions, since dpm values (controls) for these lipids did not increase with time. Labelling values for PA and DAG therefore most likely represent steady-state values.

We did not measure the masses of the lipids. Not knowing the specific radioactivities of the lipids, effects of the drugs at the precursor level are not detectable. Therefore, the precise mechanism for the observed drug effects cannot be unequivocally deduced in experiments such as ours. These reservations have been taken into account in the following discussion.

Effect of Chlorpromazine–Platelet Interaction Time

The present study shows that the effects of chlorpromazine on platelet glycerolipid metabolism are greatly dependent upon the chlorpromazine–platelet interaction time. At 30 min of the time-course in Fig. 2, labelling of DAG, TAG, and PC by ^{3}H-labelled glycerol and palmitic acid is strongly inhibited. At 2 hr, chlorpromazine actually stimulates the incorporation of the same precursors into both DAG and TAG, while the inhibitory effect on incorporation into PC is lost. Both the initial inhibitory effect of chlorpromazine and the later stimulatory effect occur at one of the steps prior to the generation of DAG since in both cases, the total incorporation of [^{3}H]glycerol through DAG as estimated by the pooled labelling of DAG, TAG, and PC was significantly affected by the drug.

Kolesnick and co-workers [15] showed that another phenothiazine, trifluoperazine, could stimulate degradation of PC and sphingomyelin. Similarly, with fairly long incubation times, one could therefore envision that at least some of the time-dependent effects were due to redistribution of radiolabel, e.g. through the activation of lipases or phospholipases. Incubation of platelets prelabelled with [^{3}H]glycerol over 2 hr did not produce any significant changes in the radioactive content of PI, PC, or TAG. In our experiments, these were the only lipids which accumulated sufficient amounts of radioactivity to be potential precursors of a redistribution reaction. The results regarding the potential phospholipase/lipase products PA and DAG were not statistically conclusive. However, the ^{3}H labelling of PA and DAG remained low ($\leq$200 dpm), and the time-courses were practically identical to the control time-courses. We therefore conclude that the time dependencies are not due to an induction of a redistribution of radioactivity by chlorpromazine at the late time points.

The time dependence was shown here not to depend on the preincubation time itself, nor did it depend on the presence of radiolabelled precursors during the incubation time. Therefore, it must be the result either of changes within the platelets produced by prolonged incubation with chlorpromazine or by the temporal distribution of chlorpromazine itself.

Trifluoperazine, another phenothiazine, stimulates acyltransfeases in cultured human fibroblasts in a time-dependent manner as shown by Maziére and co-workers [8]. Maximal effects of trifluoperazine required 6-hr preincubation with the drug, a result very similar to the time dependency observed in Figs. 2 and 4 in the present study. The acyltransferase reactions may therefore be good candidates when the mechanism of the time-dependent stimulation of [^{3}H]glycerol/palmitic acid incorporation through DAG is considered, but the present study is not conclusive in this regard.

Pathway Specificity of Chlorpromazine and Propranolol Effects on the Incorporation of Isotope-labelled Precursors into Platelet Glycerolipids

In the second part of this study, we show that chlorpromazine and propranolol affect the incorporation of isotope-labelled precursors into PC in a qualitatively different way, depending on whether the precursors chosen are incorporated through the CDP choline or the DAG-generating pathway (Fig. 1), respectively. Labelling of PC by [^{3}H]glycerol/palmitic acid was inhibited in a dose-dependent manner by chlorpromazine and propranolol, while labelling by [^{14}C]choline or [^{32}P]P_i was not affected by any of the drugs. Since similar results were obtained using two more or less unrelated drugs, the phenothiazine antipsychotic chlorpromazine and the β-blocking agent propranolol, the observed results are not the specific feature of a single drug, but must be due to a more general feature of lipid metabolism in platelets. The time-courses for 25 μM chlorpromazine obtained with [^{3}H]glycerol or [^{3}H]palmitic acid also showed the opposite tendency as compared to the time-course with [^{32}P]P_i, although the increasing inhibitory effect of chlorpromazine on labelling of PC by [^{32}P]P_i was not significant.

Chlorpromazine also inhibited the total incorporation of [^{3}H]glycerol through DAG, as measured by the combined radioactivities of DAG, TAG, and PC. DAG is also an intermediate for the synthesis of phosphatidylethanolamine, which was not measured. However, since the observed values represent the labelling of two separate end-products (TAG and PC) derived from DAG, together with the labelling of the DAG itself, we choose to regard the measured values in Table 2 as reliable estimates of the incorporation of [^{3}H]glycerol into platelet lipids through DAG. We thus conclude that the observed inhibition of labelling of PC by [^{3}H]glycerol and [^{3}H]palmitic acid by chlorpromazine is not due to a specific inhibition of the CDP choline pathway (Fig. 1), but is rather the result of an inhibition of the DAG-generating pathway affecting all the

lipids derived from DAG. Chlorpromazine is known to inhibit PAP in other cells [1]. Propranolol, another inhibitor of PAP [1], had a similar effect in our experiments, although in this case inhibition was not significant. It is therefore likely that the observed inhibition is due to an inhibition of PAP. The increase of the steady-state level of PA (Fig. 6) caused by both drugs fits this model well, as does the inhibition of incorporation of [^{3}H]glycerol through DAG. However, as we did not measure PAP activity or calculate the flow rates of PA and DAG, the exact mechanism of the inhibitory effect can not be deduced from the present results.

General Discussion and Speculations

The [^{3}H]glycerol used was labelled in the 2-position. The ^{3}H-labelled *sn*-2 hydrogen atom could be exchanged with an unlabelled hydrogen by the shuttling between glycerol-3-phosphate and dihydroxyacetonephosphate in the reversible glycerol-3-phosphate dehydrogenase (*sn*-glycerol 3-phosphate:NAD$^+$ 2-oxidoreductase, EC 1.1.1.8) reaction (reaction 8, Fig. 1). This could reduce the specific radioactivity of [^{3}H]glycerol over time and thus complicate the interpretation of data. Effects of the drugs on the exchange process could, in principle, be responsible for some of the observations made. Furthermore, we have not measured the specific radioactivities of any of the early intermediates such as [^{3}H]-glycerol-3-phosphate, or [^{3}H]palmitoyl CoA, [^{14}C]choline phosphate or [^{32}P]ATP. Thus, in principle, any of the effects observed could be due to the interaction of the drugs with processes affecting the specific radioactivites of these intermediates, prior to their entry into lipid metabolism. However, the following factors argue against this: 1. Experiments with [^{3}H]glycerol, [^{3}H]palmitic acid, and [^{14}C]choline were performed in the continuous presence of excess exogenous radiolabelled precursor. Thus, although the specific radioactivity of [^{3}H]glycerol/palmitic acid could have changed somewhat after uptake into the platelets, there was a continuous replenishment with fresh precursor during the experiment, assuring that the overall change in specific radioactivity was minimal, 2. In all the experiments, the use of at least two different isotope-labelled precursors yielded similar results. Any effects of the drugs on the specific radioactivities of either the precursors themselves or on their early intermediates would have resulted in the divergence of results obtained with the two different precursors. This was never observed, and in the case of the ^{3}H-labelled precursors, we were able to show that their incorporation into PC was actually functionally coupled (Table 1). 3. Any effects of the drugs at a step prior to a precursor's entry into lipid metabolism would affect all the monitored lipids uniformly. However, in the dose–response experiments, the drugs inhibited the labelling of DAG and its derivatives by [^{3}H]glycerol and [^{3}H]palmitic acid (Fig. 5, Table 2), while strongly stimulating the ^{32}P labelling of PI (Fig. 5, insets). This is not compatible with an effect at the precursor level. Furthermore, while the drugs had no effect on the labelling of PC by [^{32}P]P_i (Fig. 5), both drugs strongly stimulated the labelling of PI (Fig. 5, insets). Based on these arguments, we conclude that in all likelihood, the present results were not produced by changes in the specific radioactivities of the pools of any of the radiolabelled precursors or their early intermediates.

In addition to incorporation through *de novo* synthesis of DAG, [^{3}H]palmitic acid could also be incorporated into PC through remodelling reactions. In this case, a fatty acid of a pre-existing PC molecule is exchanged with another fatty acid through a number of mechanisms. Exchange of the fatty acid in the *sn*-2 position is well known, but exchange can occur in the *sn*-1 position as well through several mechanisms [16, 17], e.g. by the successive actions of phospholipase A_1 and acyltransferase (reaction 7, Fig. 1). However, in the time experiments, results obtained with [^{3}H]palmitic acid were very similar to those obtained with [^{3}H]glycerol, which cannot be incorporated through remodelling. Furthermore, we show in the dose–response experiments (Fig. 5, Table 1) that [^{3}H]glycerol and [^{3}H]palmitic acid are functionally coupled with regard to their incorporation into PC. The dose–response relationships for the effects of both drugs on the labelling of PI by the same precursors are also very similar for [^{3}H]glycerol and [^{3}H]palmitic acid, respectively (Fig. 5, insets). We take this as a strong indication that in these particular experiments, the two precursors are incorporated mainly through a common pathway, i.e. through *de novo* synthesis.

Incorporation of precursors into PC through the DAG-generating pathway can be inhibited by 60–90% without affecting the incorporation of [^{14}C]choline or [^{32}P]P_i through the CDP–choline pathway. One may therefore speculate that CDP–choline from the CDP–choline pathway is generally coupled with DAG that is not derived from *de novo* synthesis in platelets. Another possibility may be that DAG is synthesised in two separate compartments, one of which is inaccessible to the exogenously added precursors.

In conclusion, we show in the present study that drug–platelet interaction time is of essential importance when effects of chlorpromazine on platelet lipid metabolism are to be investigated. For some lipids, we observed a whole range of effects: from a complete block, through no effect at all, to a large stimulation of incorporation of isotope-labelled precursors. Some of the effects of chlorpromazine on lipid metabolism observed in short-term experiments may therefore be of little physiological relevance. Furthermore, we show that the observed effect of propranolol and chlorpromazine on the incorporation of isotope-labelled precursors into platelet PC is highly dependent upon the pathway through which the specific precursor used is incorporated, and that the diacylglycerol-generating pathway can be manipulated independently of the CDP choline pathway. These results have implications for the interpretation of data regarding drug effects on lipid metabolism in general, and for the interpretation of data regarding incorporation of isotope-labelled precursors into platelet lipids in particular.

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References

1. Jamal Z, Martin A, Gomez Munoz A and Brindley DN, Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol. *J Biol Chem* **266:** 2988–2996, 1991.
2. Martin A, Hopewell R, Martin-Sanz P, Morgan JE and Brindley DN, Relationship between the displacement of phosphatidate phosphohydrolase from the membrane-associated compartment by chlorpromazine and the inhibition of the synthesis of triacylglycerol and phosphatidylcholine in rat hepatocytes. *Biochim Biophys Acta* **876:** 581–591, 1986.
3. Brindley DN, Allan D and Michell RH, The redirection of glyceride and phospholipid synthesis by drugs including chlorpromazine, fenfluramine, imipramine, mepyramine and local anaesthetics. *J Pharm Pharmacol* **27:** 462–464, 1975.
4. Pelech SL, Jetha F and Vance DE, Trifluoperazine and other anaesthetics inhibit rat liver CTP: phosphocholine cytidylyltransferase. *FEBS Lett* **158:** 89–92, 1983.
5. Pelech SL and Vance DE, Trifluoperazine and chlorpromazine inhibit phosphatidylcholine biosynthesis and CTP:phosphocholine cytidylyltransferase in HeLa cells. *Biochim Biophys Acta* **795:** 441–446, 1984.
6. Rabkin SW, Effects of chlorpromazine and trifluoperazine on choline metabolism and phosphatidylcholine biosynthesis in cultured chick heart cells under normoxic and anoxic conditions. *Biochem Pharmacol* **38:** 2349–2355, 1989.
7. Hoshi K and Fujino S, Difference between effects of chlorpromazine and perphenazine on microsomal phospholipids and enzyme activities in rat liver. *J Toxicol Sci* **17:** 69–79, 1992.
8. Maziere C, Maziere JC, Mora L, Auclair M and Polonovski J, Trifluoperazine increases fatty acid turnover in phospholipids in cultured human fibroblasts. *Lipids* **23:** 419–423, 1988.
9. Vance DE, Glycerolipid biosynthesis in eukaryotes. In: *Biochemistry of Lipids, Lipoproteins and Membranes* (Eds. Vance DE and Vance JE), pp. 153–181. Elsevier, Amsterdam, 1996.
10. Tysnes OB, Verhoeven AJ and Holmsen H, Studies on the preferential incorporation of [^{3}H]glycerol over [^{32}P]phosphate into major phospholipids of human platelets. *Biochim Biophys Acta* **930:** 338–345, 1987.
11. Tysnes OB, Aarbakke GM, Verhoeven AJ and Holmsen H, Thin-layer chromatography of polyphosphoinositides from platelet extracts: Interference by an unknown phospholipid. *Thromb Res* **40:** 329–338, 1985.
12. Lages B, Scrutton MC and Holmsen H, Studies on gel-filtered human platelets: Isolation and characterization in a medium containing no added Ca^{2+}, Mg^{2+}, or K^+. *J Lab Clin Med* **85:** 811–825, 1975.
13. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37:** 911–917, 1959.
14. Tysnes OB, Verhoeven AJ and Holmsen H, Phosphate turnover of phosphatidylinositol in resting and thrombin-stimulated platelets. *Biochim Biophys Acta* **889:** 183–191, 1986.
15. Kolesnick RN and Hemer MR, Trifluoperazine stimulates the coordinate degradation of sphingomyelin and phosphatidylcholine in GH_3 pituaitary cells. *J Biol Chem* **264:** 14057–14061, 1989.
16. MacDonald JIS and Sprecher H, Phospholipid remodeling in mammalian cells. *Biochim Biophys Acta* **1084:** 105–121, 1997.
17. Tijburg LBM, Samborski RW and Vance DE, Evidence that remodeling of the fatty acids of phosphatidylcholine is regulated in isolated rat hepatocytes and involves both the *sn*-1 and *sn*-2 positions. *Biochim Biophys Acta* **1085:** 184–190, 1991.