Biochemical Pharmacology, Vol. 60, pp. 1267–1277, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00445-7

The Stimulatory Effects of Cationic Amphiphilic Drugs on Human Platelets Treated with Thrombin

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ABSTRACT. The actions of eight cationic amphiphilic drugs on human platelets displayed three different effects according to drug concentration ranges. At lower concentrations (below ~25 μM), the drugs stimulated secretory responses induced by 0.2 U/mL of thrombin, while at concentrations in the 25–50 μM range they inhibited these responses. Above 50–100 μM, the drugs caused permeabilization of the platelet plasma membrane as measured by leakage of cytoplasmic adenine nucleotides. The effects of these agents on phosphoinositide metabolism were monitored in platelets prelabeled with ^{32}P-inorganic phosphate, such that phosphatidic acid (PA), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2), but not phosphatidylinositol (PI), were labeled to equilibrium. In unstimulated platelets, the level of labeled PA decreased slightly (about 25%), with corresponding increases in PIP_2 labeling up to drug concentrations of about 50 μM. In contrast to the relatively small changes in PI and PIP_2, the levels of labeled PIP, precursor to PIP_2, increased 2- to 4-fold in both resting and thrombin-treated platelets from 5 μM up to about 50–100 μM of drugs and remained elevated throughout the permeabilization concentrations. [^{32}P]PA increased 20-fold over control upon thrombin activation and 5–30 μM of drugs caused [^{32}P]PA to increase 30–37 times over that seen in control, resting platelets; the concentration of drugs that potentiated thrombin-induced [^{32}P]PA elevation corresponded to that causing the potentiation of platelet secretion. Higher drug concentrations decreased [^{32}P]PA elevation. [^{32}P]PIP_2 levels increased about 25% in response to thrombin treatment alone; low concentrations of drugs led to another 25% elevation. A significant decrease in [^{32}P]PIP_2 was seen above 30 μM, corresponding to inhibition of platelet secretion. Concentrations of 5–30 μM of several psychoactive agents, both neuroleptics and antidepressants, potentiated the thrombin-induced activation of platelets as measured by dense granule secretion and increased turnover of phosphoinositides. Remarkably, all of the drugs increased the levels of PIP even in resting platelets, indicating that they have common effects apart from the specific receptor interactions currently attributed to them. These common effects, e.g. an increase in membrane fluidity such as is known to be caused by amphipathic agents, may be in part responsible for the observed overlapping psychotrophic effects of tricyclic antidepressants and phenothiazines. BIOCHEM PHARMACOL **60**;9:1267–1277, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. phenothiazines; tricyclic antidepressants; phosphoinositide metabolism; receptor potentiation; membrane alteration

Tricyclic antidepressants and psychoactive phenothiazines, collectively referred to as cationic amphiphilic drugs, have been intensively investigated and used in psychiatry for the past four decades, but the underlying biochemical mechanisms of their action are still poorly understood. The phenothiazines are thought to act by binding to and blocking dopamine D_2 receptors as well as α_2-adrenergic, muscarinic cholinergic, and histamine H_1 receptors; many also block serotonin receptors. The tricyclic antidepressants are considered to act by inhibition of serotonin and noradrenaline uptake, thus enhancing the effect of these neurotransmitters, but not all agents that stimulate serotonergic or noradrenergic transmission are effective antidepressants [1]. Some antidepressants are not only serotonin uptake blockers, but also blockers of $5HT_{1A}$ and $5HT_{2A}$ receptors [2].

The presumed targets of both of these classes of drugs are so diverse that it seems unlikely that specific interaction of drug with any one of them is truly the sole mechanism of action. In any case, the most obvious, common feature of these amphiphilic agents is that they are able to cross the blood–brain barrier. It seems highly unlikely that such small molecular weight, lipid-soluble substances would remain exclusively at the cell surface and react only with specific receptors. Distribution into the plasma membrane and intracellular membranes would be expected. Accordingly, a number of investigations have been carried out on postreceptor effects of phenothiazine-type drugs in cells, especially blood platelets, and mostly inhibitory effects have been reported in the literature [3–8]. Numerous studies have reported various effects on phosphoinositide

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Received 16 November 1999; accepted 14 March 2000.

metabolism [8–17]. We have previously reported that concentrations of CPZ* and TFZ over 50–100 μM cause leakage of small molecular weight substances such as ATP from the cell without concomitant leakage of macromolecules such as lactate dehydrogenase [11, 18]. Loss of essential cytoplasmic components could be responsible for the inhibitory effects reported in numerous studies in which drug concentrations ranging from 50–2000 μM have been used [3–15].

In order to define carefully the concentration range at which this leakage begins, a dose titration of the effects of phenothiazines from concentrations as low as 5 to 10 μM on thrombin-induced platelet activation was carried out in this study. Surprisingly, stimulation of normal thrombin activation was observed at these concentrations of drugs, similar to earlier findings reported by Rao for TFZ [19]. Because of the diverse effects reported in individual studies using various concentrations of different amphipathic agents, we have made this a comparative study of 8 substances, 5 phenothiazines and 3 antidepressants, as well as a phenothiazine sulfoxide, a cationic, and an anionic detergent on activation parameters in platelets.

We report in this paper that similar stimulatory effects were seen with low concentrations of these various neuroleptic phenothiazines and tricyclic antidepressants on thrombin-induced platelet activation, but no effects were seen with low concentrations of the sulfoxide or detergents. At high enough concentrations, all active agents caused lysis and leakage of cytoplasmic adenine nucleotides.

MATERIALS AND METHODS

Platelet Isolation

Blood was obtained from regular donors at the blood bank (Haukeland Hospital, Bergen, Norway), who claimed to have taken no medication for the previous ten days. Thirty-six milliliters of blood was mixed with 6 mL "acid citrate dextrose" (86.7 mM trisodium citrate, 73.3 mM citric acid, 100 mM dextrose) as anticoagulant, and then centrifuged at 535 *g* for 6 min at ambient temperature. Platelet-rich plasma was collected and centrifuged again at 1200 × *g* for 10 min (ambient temperature) to pellet the platelets. Two-thirds of the supernatant plasma was discarded and the platelets resuspended in the remainder of the plasma. This suspension was incubated for 60 min at 37° with [^{32}P]orthophosphate (0.1 mCi/mL, carrier free, Amersham, code PBS-11). Subsequently, the platelets were gel-filtered into a phosphate- and Ca^{2+}-free Tyrode's solution (pH 7.25) containing 0.2% (w/v) BSA and 5 mM glucose [20]. Final platelet counts in gel-filtered platelets were adjusted to 3.5 × 10^8 cells/mL. The gel-filtered platelets for measurement of drug-induced permeabilization were also prepared in the same manner, except that the concentrated platelet-rich plasma was incubated with 1 μM [2-^{3}H]adenine (specific radioactivity 3.5 Ci/mmol, New England Nuclear, code NET-350).

Incubation with Drugs

The phenothiazines used were: PPSO (Schering-Plough), PCP and PMZ (Rhone-Poulenc Pharma Norden A/S), TFZ (Sigma), and TPZ (Bristol-Myers Squibb). The tricyclic antidepressants used were: AMI, NTR, and IMI (Sigma). One cationic detergent (CTAB; Merck) and one anionic detergent (DSS; Sigma) were also tested. The drugs and detergents were dissolved in 0.9% NaCl and stock solutions were stored cold in the dark for not more than 2 months. Platelets were preincubated for 3 min with the drugs at 37° before the addition of 0.2 U/mL of thrombin or saline. After 90 sec of incubation, samples were prepared for analysis of phospholipids, nucleotides, and secretions.

Phospholipid Extraction

Samples from the incubation mixture were collected in 4 vol. of chloroform/methanol/conc.HCl (20:40:1 by vol.; 0°) and processed further as described elsewhere [21]. The separation of phospholipids was performed by thin layer chromatography on silica gel plates (art. 5553, Merck) in chloroform/methanol/20% methylamine in water (60:36:10 by vol.) [22]. The spots were localized by overnight autoradiography with Fuji x-ray film and then scraped off, and the ^{32}P content of the phospholipids was determined by liquid scintillation counting.

Secretion

Samples were pipetted into precooled Eppendorf tubes in ice containing 0.14 vol. of 1 M formaldehyde/50 mM EDTA in order to stop secretion [23]. These fixed samples were centrifuged (not totals) for 2 min at 12,000 *g* at room temperature. One hundred microliters of the supernatants was mixed with 100 μL of 10 mM EDTA in 86.4% ethanol for determination of nucleotides by a firefly luminescence method [24]. The total amounts of ATP and ADP in gel-filtered platelets were determined in EDTA–ethanol extracts of non-centrifuged samples. Another 100 μL aliquots of the supernatants were mixed with 0.1% Triton X-100 for determination of β-hexosaminidase with 4-methyl-umbelliferyl *N*-acetyl-β-D-glucosaminide as substrate [23]. The total activities of the acid hydrolase were determined in Triton X-100 extracts of non-centrifuged platelets.

Permeabilization

The appearance of cytoplasmic substances in the extracellular medium was determined as a sign of permeabilization

* Abbreviations: AMI, amitryptiline; CPZ, chlorpromazine; CTAB, cetyltrimethylammonium bromide; DAG, diacylglycerol; DSS, dioctyl sulfosuccinate; IMI, imipramine; NTR, nortriptyline; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PCP, prochlorperazine; PPSO, perphenazine sulfoxide; PMZ, promethazine; TFZ, trifluoperazine; and TPZ, triflupromazine.

in the same supernatant samples taken for secretion. The extracellular appearance of [2-^{3}H]adenine nucleotides was measured by high-voltage paper electrophoresis of EDTA–ethanol extracts of the supernatant secretion samples taken of platelets preincubated with [2-^{3}H]adenine [7]. The total radioactivities of ATP and ADP were determined in EDTA–ethanol extracts of non-centrifuged platelets. The % secretion or permeabilization was determined by the following formula:

$$\frac{\text{Amount/activity in supernatant}}{\text{Amount/activity in total}} \times 100$$

RESULTS

The effects of cationic amphiphilic drugs on the parameters determined in the present study varied considerably among platelets from different blood donors. However, the parameters measured always varied with the concentration of a given drug in the same pattern among the donors. The results are therefore presented as single experiments, each of which has been performed with platelets from at least 3 donors.

Release of Low Molecular Weight Cytoplasmic Substances by Cationic Amphiphilic Drugs at High Concentrations

Platelet cytoplasmic adenine nucleotides were specifically labeled by incubation of platelets in plasma with [2-^{3}H]adenine for 60 min [7]. After gel filtration of platelets into Tyrode's solution to remove plasma and unincorporated radioactive marker, the platelets were preincubated for 3 min at 37° with AMI, NTR, IMI, or PMZ at concentrations greater than 50–100 μM. Saline or thrombin was added and after another 90-sec incubation, the cells were fixed with EDTA–formaldehyde and centrifuged to separate extracellular and cellular compartments. As much as 60–70% of total radiolabeled nucleotides was found in the extracellular medium for all drugs, except for IMI, at concentrations of 200 μM (Fig. 1). IMI did not cause the release of radioactive adenine nucleotides in the absence of agonist, but did cause about a 5–20% release in the presence of agonist at 100 μM.

Effect of Cationic Amphiphilic Drugs on Thrombin-Induced Secretion of Storage Granule Contents

Markers used to measure the secretion of dense granules were the mass of stored ATP + ADP by a firefly luciferase assay [25]; these nucleotides do not incorporate radioactive label in the course of 1- to 2-hr incubation as do the metabolically active cytoplasmic nucleotides. The lysosomal secretion marker was β-hexosaminidase. TFZ, TPZ, PCP, AMI, and NTR potentiated thrombin-stimulated dense granule and lysosome secretion at concentrations between 5 and 20 μM as shown in Figs. 2A and 3B, respectively. The psychotherapeutically inactive phenothiazine PPSO, the cationic detergent CTAB, and the anionic detergent DSS had no significant effect on the secretory processes at these low concentrations. An increase in the drug concentrations above 20 μM caused a progressive inhibition of the two secretory processes, which reached its lowest level around 50–100 μM. CTAB inhibited both secretory processes in this concentration range, while PPSO and DSS had no effects. Concentrations of the drugs and CTAB above 50–100 μM in the presence of thrombin produced an increase in the extracellular concentration of both secretion markers, while PPSO and DSS had no effect (Figs. 2A and 3B). This latter "release" of secretion markers occurred in the same concentration range as did the appearance of cytoplasmic nucleotides shown in Fig. 1.

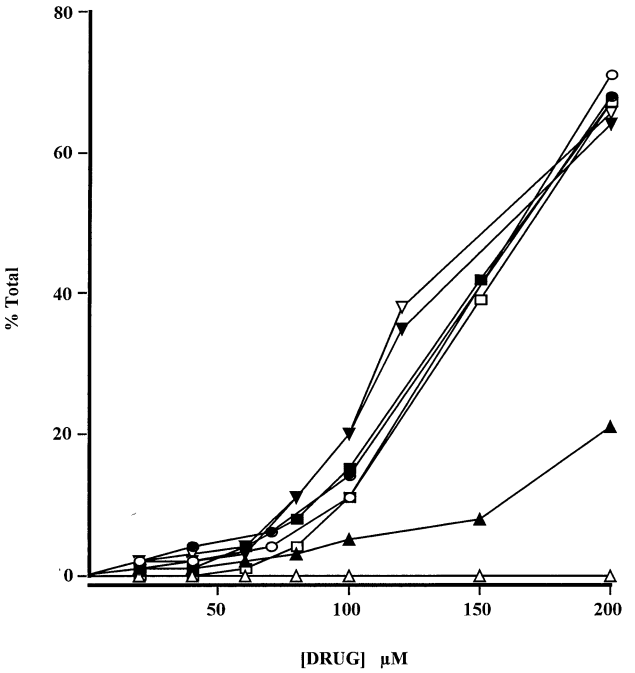

FIG. 1. Effect of drug concentration on release of non-secretable, cytoplasmic adenine nucleotides (prelabeled with [^{3}H]adenine) into platelet supernatants. Platelets were preincubated for 3 min with PMZ (○, ●), NTR (▽, ▼), IMI(△, ▲), or AMI(□, ■) prior to addition of saline (open symbols) or thrombin (closed symbols). Platelets were then centrifuged and the % radioactive ATP and ADP in the supernatants compared to total platelets were estimated as described under Methods. The experiment is representative of 3 or more experiments with different donors.

In the absence of agonist (thrombin was replaced by saline in these experiments), no or little secretion was observed at 0–50 μM of the active drugs, which showed that these agents did not induce secretion per se (Figs. 1 and 3A). At drug concentrations above 50–100 μM, ATP and ADP began to appear extracellularly both in the absence and presence of thrombin, showing that this release was not a specific secretory response. PPSO and DSS had no effect on either unstimulated or activated responses.

The pattern of stimulation of secretion at low concentrations of active drug, followed by inhibition of secretion at intermediate concentrations of drug and an indiscrimate

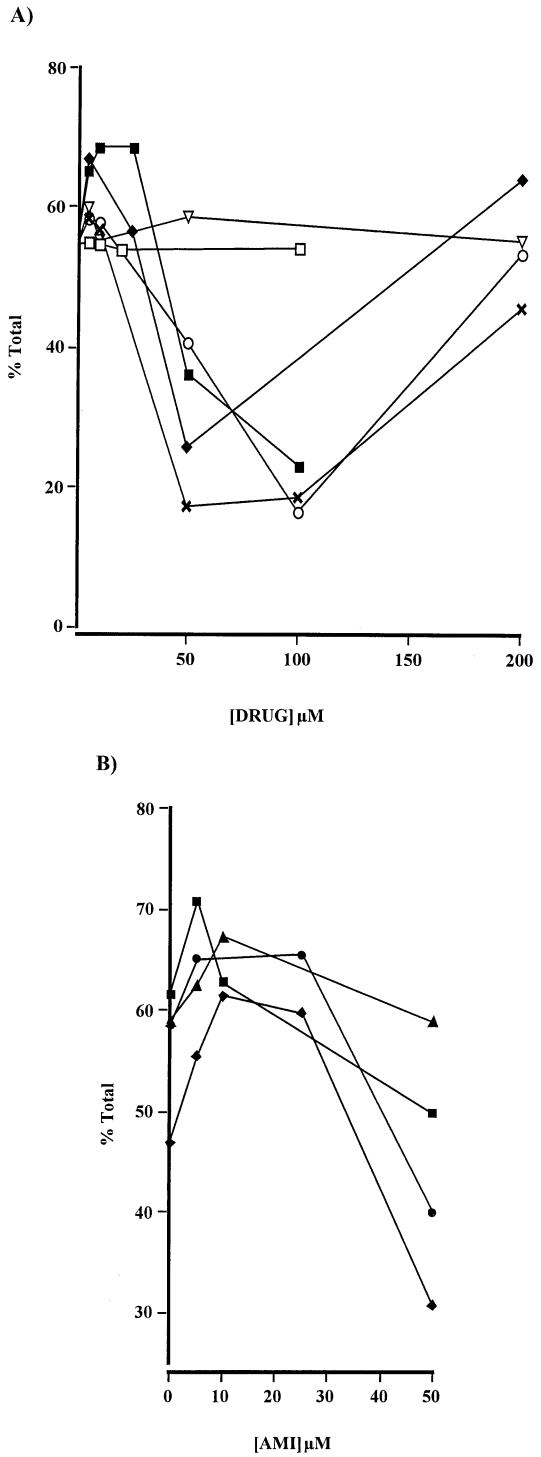

FIG. 2. Effect of drug and detergent concentrations on the appearance of extracellular ATP and ADP (mass) in platelet supernatants after treatment with 0.2 U/mL of thrombin for 90 sec. (A) The % ATP and ADP (mass) released by thrombin-treated platelets at 0 drug concentration has been corrected by subtraction of nucleotides in supernatants of platelets not treated with thrombin. The different agents used were TFZ (◆), TPZ (✕), AMI (■), PPSO (□), CTAB (○), and DSS (▽). The experiment is representative of 3 or more experiments with different donors. (B) Release of ATP and ADP mass is shown for one of the drugs, AMI, from 4 different donors at the concentration range of 0–50 μM to show reproducibility in the pattern of potentiation.

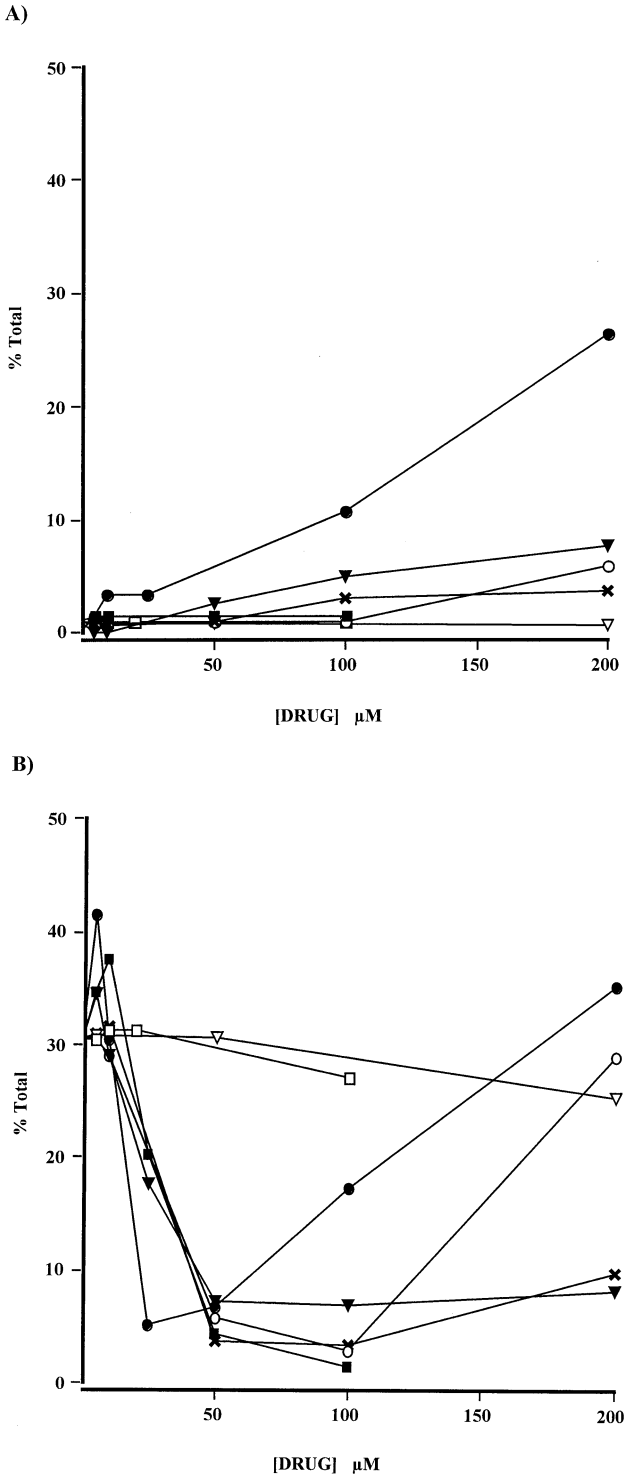

FIG. 3. Effect of drug and detergent concentration on β-hexosaminidase release from control platelets (A) and thrombin-treated platelets (B). Platelets were preincubated with drug for 3 min before addition of saline or thrombin 0.2 U/mL for 90 sec. Acid hydrolase was measured in platelet supernatants after 90 sec. Symbols represent: TPZ (✕), PCP (●), AMI (■), NTR (▼), PPSO (□), CTAB (○), and DSS (▽). Representative of 3 different donors.

release at the high concentrations of drug, were qualitatively similar for both dense granule and lysosomal extracellular marker appearance. The quantitative difference between the two types of granule secretion arose because β-hexosaminidase is localized in secretory granules as well as being bound to another membrane compartment, whereas adenine nucleotides are localized both in secretory granules and in the cytoplasm. Thus, the appearance of adenine nucleotides extracellularly is due both to secretion from granules with agonist activation and leakage from cytoplasm upon plasma membrane permeabilization.

Effect of Cationic Amphiphilic Drugs on [^{32}P]PA and [^{32}P]Phosphoinositides in Platelets Treated without and with Thrombin

[^{32}P]P_i-prelabeled platelets were incubated with increasing concentrations of drugs for 3 min followed by a 90-sec incubation with and without 0.2 U/mL of thrombin, and the levels of ^{32}P-labeled phosphatidic acid and phosphoinositides were determined. The results are expressed in percent of the radioactivity in the appropriate phospholipid in control cells in the absence of drugs (Figs. 4–7).

Phosphatidic Acid

The levels of [^{32}P]PA were low in non-stimulated platelets (around 400 cpm/mL of platelets in our experiments), but were increased about 20-fold by incubation with thrombin (compare the ordinates of Fig. 4B with 4A where no drug is present). There was no systematic effect on [^{32}P]PA with increased concentrations of any drug up to 50 μM in control platelets without thrombin, while further increases in the concentration of NTR, TFZ, PCP, and AMI caused a progressive increase in the level of [^{32}P]PA to about 2-fold at 200 μM; PPSO, CTAB, DSS, and PMZ were without effect (Fig. 4A). NTR, TFZ, PCP, and AMI had concentration-dependent, opposing effects on the thrombin-induced formation of [^{32}P]PA: (i) a 50–80% increase over the 20-fold increase produced by thrombin alone between 5 and 25 μM of drug (Fig. 4A); and (ii) a progressive decrease from this peak level as the drug concentration was further increased to 200 μM (Fig. 4B). PPSO and DSS had no effects on the thrombin-induced increase in [^{32}P]PA over the entire 0–200 μM range, while CTAB had no effect over that of thrombin alone at low concentrations; however, high detergent concentrations resulted in a progressive decrease in the thrombin-induced [^{32}P]PA formation (Fig. 4B).

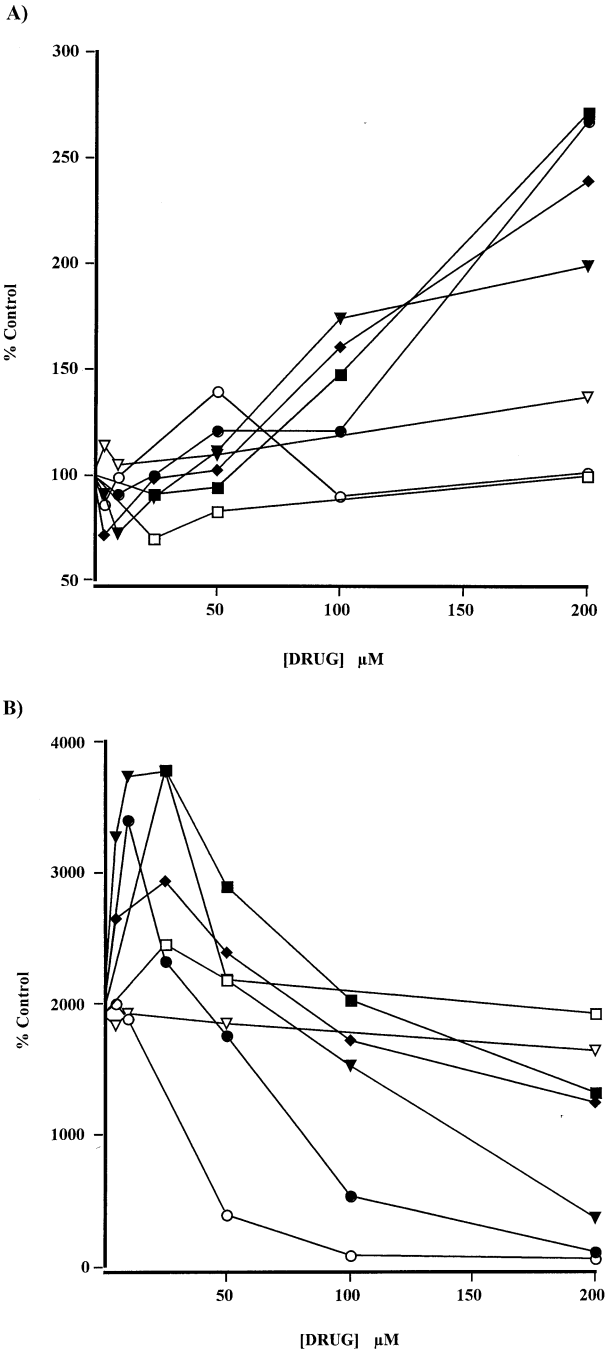

FIG. 4. Effect of drug and detergent concentration on the levels of [^{32}P]PA in platelets. Platelets prelabeled with ^{32}P-inorganic phosphate were preincubated for 3 min with drug or detergent before addition of saline (A) or 0.2 U/mL of thrombin (B). After 90 sec, the incubations were stopped and the lipids extracted as described under Methods. Radioactive PA was determined after TLC separation and plotted as % of drug-free control without thrombin, which contained 247 cpm. The symbols represent: TFZ (◆), PCP (●), AMI (■), NTR (▼), PPSO (□), CTAB (○), and DSS (▽). Representative of 3 donors.

Phosphatidylinositol

An increase in the concentrations of the psychoactive drugs PPSO, CTAB, and DSS caused a small decrease in the level of [^{32}P]PI in the absence of thrombin (Fig. 5A). When platelets were incubated with thrombin for 90 sec in the absence of the compounds tested, the level of [^{32}P]PI increased by about 30% (compare Fig. 5, A and B, at the ordinates). Increasing the concentrations of the psychoactive drugs caused a decrease in this elevated [^{32}P]PI (Fig. 5B) which was similar to, but much less expressed than that found for [^{32}P]PA (Fig. 4B). As found for [^{32}P]PA, an

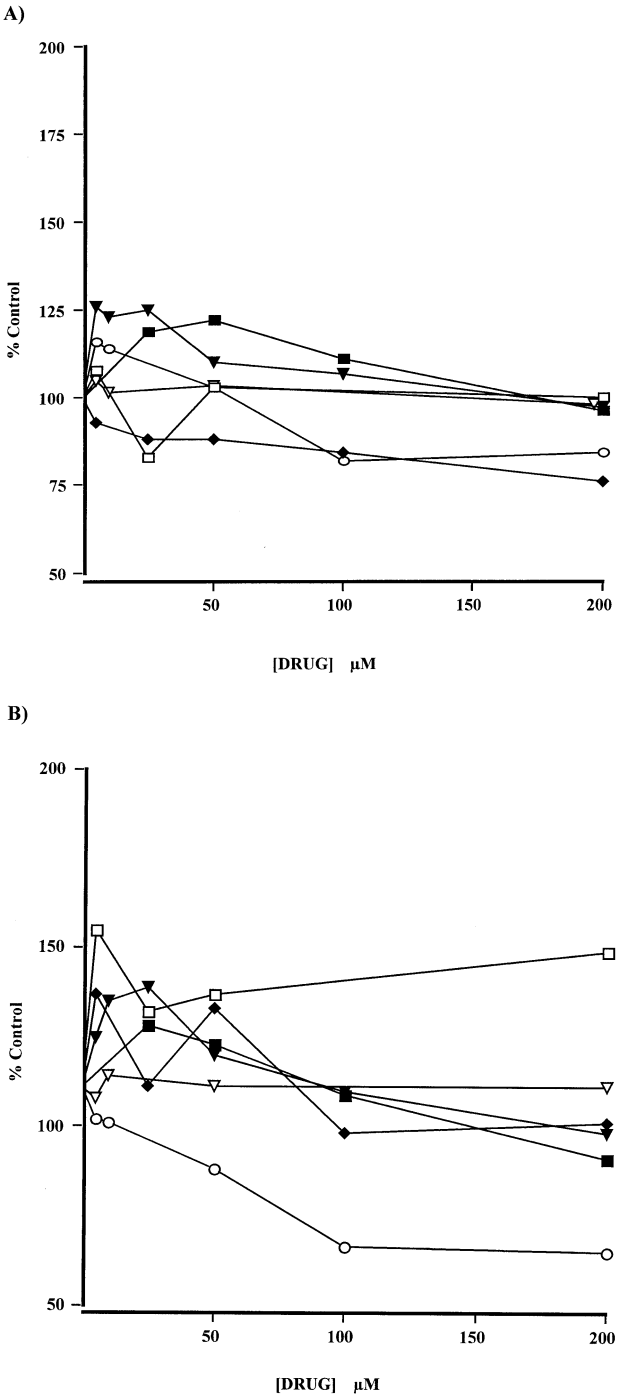

FIG. 5. Effect of drug and detergent concentration on the levels of [^{32}P]PI in control platelets (A) and thrombin-treated platelets (B). These results are from the same experiments described in Fig. 4, expressed as % radioactivity in drug-free control without thrombin, which contained 2605 cpm. The symbols are the same as in Fig. 4 (PCP, which was similar to TFZ, was not plotted to simplify the figure).

increase in the concentration of CTAB produced a progressive decrease in the level of [^{32}P]PI, while an increase in the concentration of DSS had no effect on [^{32}P]PI (Fig. 5B). Surprisingly, an increase in the concentration of PPSO, which had no effect on the thrombin-induced increase in the level of [^{32}P]PA (Fig. 4B), increased [^{32}P]PI maximally at 5 μM, a level that did not vary with any further increase in the PPSO concentration (Fig. 5B).

Phosphatidylinositol 4-phosphate

Increasing the concentration of all the psychoactive agents up to 100 μM in the absence of thrombin caused a marked increase in the level of [^{32}P]PIP, which was greatest (6-fold) for NTR and PCP, intermediate (3-fold) for PMZ, and smallest (2-fold) for TFZ and AMI; a further increase in the drug concentrations produced no further increase in [^{32}P]PIP (Fig. 6A). PPSO and DSS had no effect, while an increase in the concentration of CTAB caused a small increase in the level of [^{32}P]PIP (Fig. 6A). Treatment of platelets with thrombin in the absence of the compounds tested produced a 70% increase in the level of [^{32}P]PIP (compare the ordinates of Fig. 6; A and B). Thrombin treatment in the presence of increasing concentrations of the psychotherapeutically active drugs caused an increase in platelet PIP labeling which was maximal at 50–100 μM (Fig. 6B). Increasing concentrations of DSS and PPSO had little effect, while CTAB produced changes in [^{32}P]PIP levels in thrombin-treated platelets that were similar to those produced by the drugs (Fig. 6B).

Phosphatidylinositol 4,5-bisphosphate

The psychoactive agents had a weak, biphasic effect on the [^{32}P]PIP_2 level in unstimulated platelets. Concentrations up to 50–100 μM caused an elevation in [^{32}P]PIP_2, and even higher concentrations resulted in a decrease in [^{32}P]PIP_2, particularly for PCP and NTR (Fig. 7A). Thrombin treatment of platelets for 90 sec in the absence of drugs or detergents increased the level of [^{32}P]PIP_2 by about 40% (compare the ordinates in Fig. 7, A and B). The presence of psychoactive drugs during thrombin stimulation caused a slight increase in [^{32}P]PIP_2 at 5–10 μM, while further increases in their concentration led to a massive reduction in [^{32}P]PIP_2 to 20–40% of the level found in control platelets at 100–200 μM (Fig. 7B). PPSO, DSS, and CTAB had practically no effect on the [^{32}P]PIP_2 levels in thrombin-stimulated platelets (Fig. 7B).

DISCUSSION

Effects on Cell Permeabilization and Secretion

The effects of varying concentrations of several psychoactive cationic amphiphilic drugs on membrane integrity, secretion, and phosphoinositide metabolism in resting and thrombin-activated platelets are described in this study. We found that our earlier observations showing that CPZ and TFZ caused permeabilization and loss of small molecular weight cell components at concentrations over 50 μM in both resting and thrombin-activated platelets [7, 18] were also true for all of the other psychoactive agents tested. In this study, we chose to use a thrombin concentration of 0.2

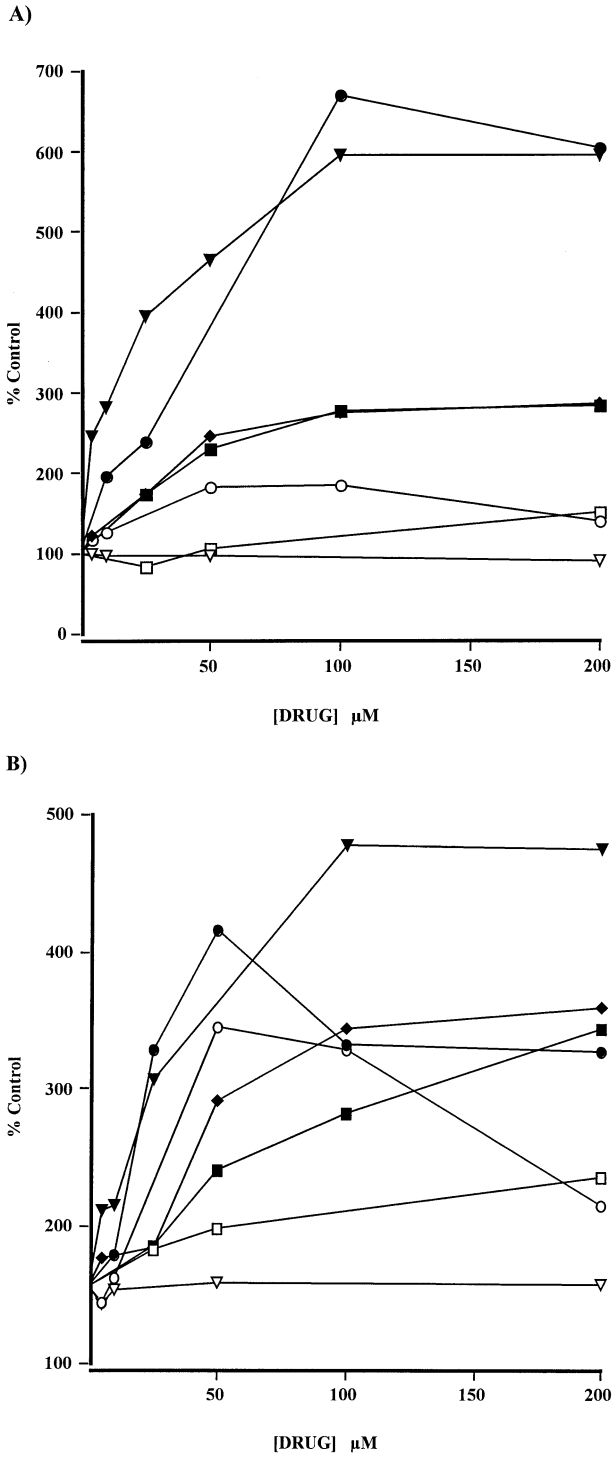

FIG. 6. Effect of drug and detergent concentration on the levels of [^{32}P]PIP in control (A) and thrombin-treated (B) platelets. The results are from the same experiments described in Fig. 4, expressed as % of radioactive PIP in drug-free control without thrombin, 5413 cpm.

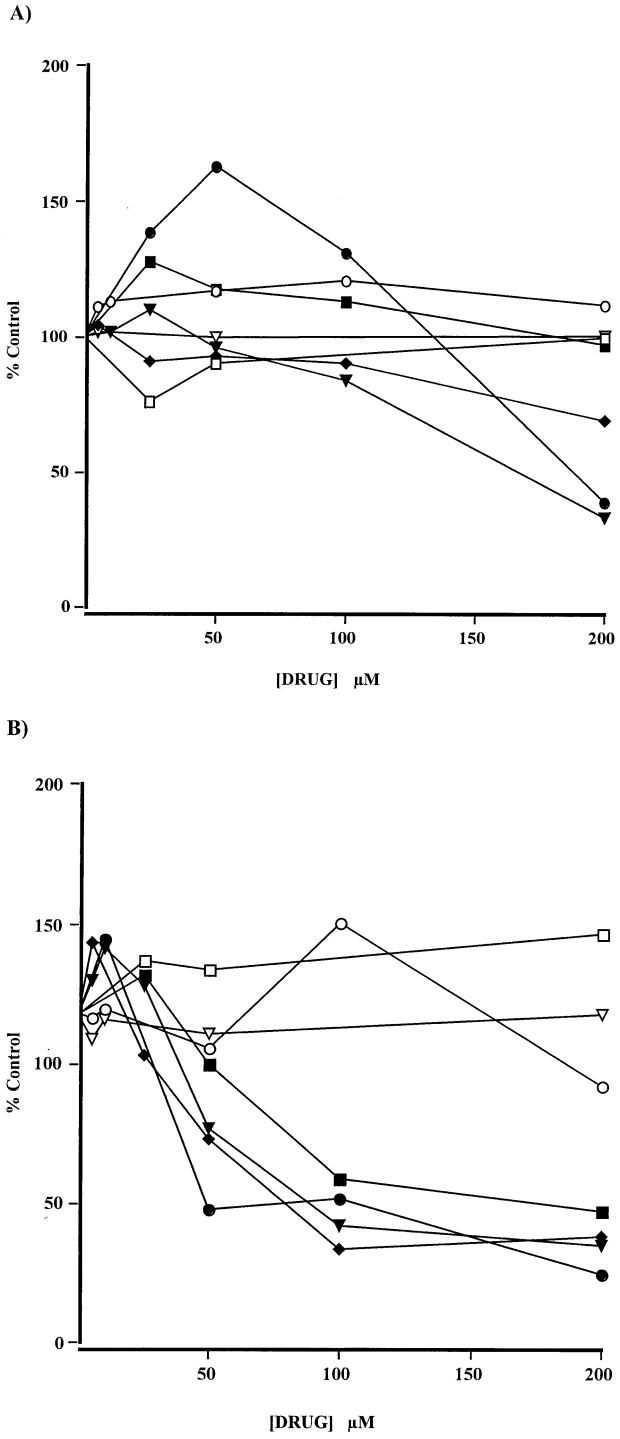

FIG. 7. Effect of drug and detergent concentration on the levels of [^{32}P]PIP_2 in platelets. The results are from the same experiments described in Fig. 4, expressed as % of radioactive PIP_2 in drug-free control without thrombin, 9834 cpm.

U/mL, which elicited somewhat less than the maximal response caused by 0.5–1 U/mL. All of the drugs caused a potentiation of platelet activation by 0.2 U/mL of thrombin at 5- to 30-μM concentrations as measured by secretion of storage nucleotides and β-hexosaminidase. Similar potentiating effects were previously reported for TFZ and CPZ in several articles [9, 11, 13, 18, 19]. No secretion was observed in resting platelets at these drug concentrations. The agonist potentiation phase was followed by inhibition of secretion with increasing drug concentration up to those concentrations where it was no longer possible to distinguish between secretion and permeabilization effects. These

permeabilizing concentrations corresponded to those observed in control platelets, which did not release cellular contents at lower drug concentrations (Fig. 1). PPSO, a psychotherapeutically inactive sulfoxide, had no stimulatory, inhibitory, or permeabilizing effects on platelets (Fig. 2). IMI and the detergent DSS had no effects on control or thrombin-activated adenine nucleotide secretion or release (Fig. 2). Although the cationic detergent CTAB did not stimulate secretion, it did inhibit secretion between 25–100 μM and caused considerable release via permeabilization above 100 μM. The permeabilizing effects of the drugs are probably attributable to similar detergent effects. We suggest that the potentiation effect of these agents at low concentrations was overlooked by many earlier investigators (including ourselves) [4–15] in favor of inhibitory effects because these latter were more consistently reproducible, either because a concentration of thrombin that elicits maximal response was used so that no further stimulation could be observed or because inhibitory concentrations of drug were used.

Effects on Polyphosphoinositide Metabolism

In discussing the general effects of psychoactive cationic amphiphilic drugs on phosphoinositide metabolism, only the effects seen under 100 μM will be considered, since the functional studies described above showed that platelet membrane integrity is compromised above that concentration. Platelets prelabeled with ^{32}P-inorganic phosphate incorporate the label into cytoplasmic ATP and into all compounds subject to ATP phosphorylations. Previous studies have established that under the labeling conditions used in these studies, the turnover of PIP and PIP_2 is so rapid that their total radioactivities are more or less representative of their relative masses, whereas PI mass is considerably greater those of PIP and PIP_2, so that its uniform labeling takes more time and this phospholipid is not labeled to equilibrium [17, 25].

The level of labeled PA in resting platelets is usually very low, and the [^{32}P]PA formed upon platelet activation as a result of ATP phosphorylation of the diacylglycerol released by phospholipase C hydrolysis of PIP_2 is representative of the mass of PA formed from PIP_2 [26]. Thrombin activation of [^{32}P]P_i-labeled platelets always results in a rapid, 15–20 fold increase in [^{32}P]PA, which remains elevated for 5–10 min [9, 15, 26]. The psychoactive drugs and detergents had little effect on phospholipase C activity in resting platelets as measured by [^{32}P]PA formation (Fig. 4A). Drug concentrations up to 25–30 μM increased the 20–fold thrombin-induced elevation of PA levels by a further 50–75% (Fig. 4B) in the same manner that it increased the secretion responses (Figs. 2 and 3). These results are consistent with increased activation of phospholipase C and potentiation of platelet activation as discussed above. The further changes in PA as well as in PI are also reasonably consistent with the changes in flux of the phosphoinositide cycle in going from activation to platelet inhibition. In platelets, very little [^{32}P]Pi is incorporated into phosphatidycholine in the labeling times used, such that phosopholipase D-catalyzed formation of radioactive PA from phosphatidylcholine is not seen [27].

PA phosphohydrolase has been reported to be inhibited by CPZ and TFZ [13, 28], but the PA formed from PIP_2-derived DAG is unlikely to be a substrate for this enzyme [29]; this 18:0/20:4 PA is more likely to be a substrate for CTP:PA cytidylyl transferase as part of a mechanism for recycling and preserving the 18:0/20:4 fatty acid composition of the molecular species that predominate in inositol phospholipids [30].

The severalfold increase in [^{32}P]PIP was the most noteworthy effect of non-permeabilizing concentrations of these drugs and cannot be explained by changes in flux of the phosphoinositide cycle resulting from increased phospholipase C activity. PIP levels were increased 300–400% even in control platelets by incubation with the psychoactive agents as shown in Fig. 6 and as reported previously [17]. Thrombin increased PIP levels about 50% in the absence of drugs, and the cationic amphipathic drugs then increased this to the same level as in resting platelets with drugs. CTAB alone of the inactive agents caused a small increase in PIP in control platelets and a 100% increase in thrombin-treated platelets; the detergent DSS and the sulfoxide compound PPSO had no effects on PIP. It can be seen from the scheme outlining phosphoinositide metabolism that the level of PIP would be altered by changes in the activity in any one of 4 different enzymes, i.e. 2 kinases and 2 phosphohydrolases (Fig. 8). PI 4-kinase has been reported to be stimulated and PIP 5-kinase inhibited by CPZ in adrenal medulla granules [31, 32].

PIP_2 was relatively unaffected by the drugs in control platelets except for PCP, which caused 50% elevation at 50 μM. A considerable decrease in [^{32}P]PIP_2 was seen above 30–50 μM drugs in thrombin-treated platelets (Fig. 7B), which was probably due to accumulation of the precursor PIP. Although there appear to be appreciable levels of PIP_2 at 50 μM of drugs, this pool of PIP_2 may be associated with the cytoskeleton [33] and unavailable for signaling.

CONCLUSIONS

The major finding we report here is that several psychoactive drugs, both neuroleptics and antidepressants, all exhibited a stimulatory effect at low drug concentrations (<30 μM) and an inhibitory effect at higher concentrations (>50 μM). The most obvious, common feature of these agents is that they are amphipathic enough to cross the blood–brain barrier. Such small molecular weight, lipid-soluble substances would easily distribute into the plasma membrane and intracellular membranes and not be limited to acting only on receptors on the outer surface of the cell. Historically, cationic amphipathic drugs such as phenothiazines have been known to have "membrane active" properties [3]. Accordingly, a number of investigations have been carried out on postreceptor effects of phenothiazine-

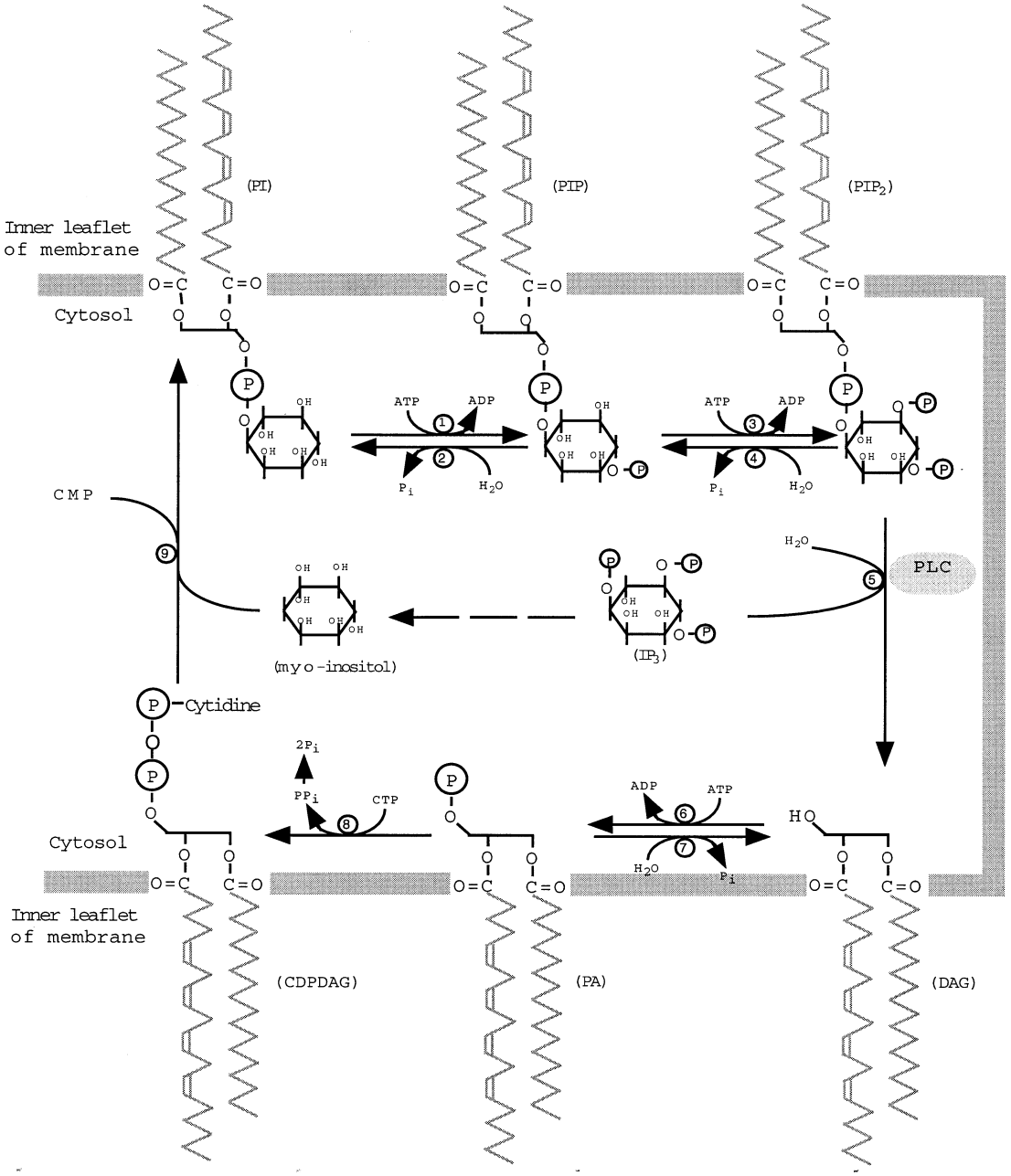

FIG. 8. The polyphosphoinositide cycle. The names of the enzymes designated by numbers 1–9 are: (1) PI 4-kinase; (2) PIP phosphomonoester hydrolase; (3) PIP 5-kinase; (4) PIP_2 phosphomonoester hydrolase; (5) phospholipase C (PLC); (6) diacylglycerol kinase; (7) PA phosphohydrolase; (8) CTP: PA cytidylyl transferase; (9) PI synthase.

type drugs in cells, especially blood platelets, and mostly inhibitory effects, with a few exceptions, have been reported in the literature [4–15]. At lower concentrations, the psychoactive agents appear to affect membrane function without causing lysis. Examples of functional disturbances in the membrane are: (i) CPZ inhibition of radioactive glycerol and palmitate incorporation into phosphatidylcholine in resting platelets [34]; and (ii) CPZ alteration of acidic phospholipid packing in monolayers as measured by the Langmuir technique* and of bilayers in liposomes as measured by solid-phase NMR [35]. Preliminary computer modeling studies of a monolayer section and CPZ or AMI show that cationic amphiphilic drugs can interact with membrane phospholipids to alter packing and surface area.† Alteration of the physical state of the cell membrane has been shown to modulate signaling in the action of steroid hormones [36] and in the induction of transcription of heat shock and several other genes by CPZ, IMI, and several local anesthetics [37]. Changes in membrane fluidity may also affect such phenomena as receptor dimer or oligomer

* Varnier A, Idsøe R, Tungodden L, Sydnes L and Holmsen H, manuscript submitted for publication.

† Tharmapathy P and Holmsen H, manuscript in preparation.

formation, which apparently is a mechanism of activation and modulation not just for growth factor receptors, but for a number of G protein-coupled receptors as well [38, 39]. We venture to suggest that tricyclic antidepressant and phenothiazine-type psychotrophic agents act not only at the level of agonist–receptor interaction as is currently postulated, but that they also affect the membrane environment in which receptors and other signaling enzymes are situated, causing activation or inhibition as the case may be. As we have shown for platelets in this study, thrombin receptor activation was potentiated and [^{32}P]PIP was elevated by low concentrations of these agents; the latter could have been caused by inhibition of PIP kinase or activation of PIP_2 phosphomonohydrolase, both of which are membrane-bound enzymes.

This work was supported by the Norwegian Research Council for Science, the Blix Fund, the EU Biomed 2 program contract no. BMH4-CT-97 2609, and the L. F. Saugstad Foundation.

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