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CHLORPROMAZINE INCREASES THE TURNOVER OF METABOLICALLY ACTIVE PHOSPHOINOSITIDES AND ELEVATES THE STEADY-STATE LEVEL OF PHOSPHATIDYLINOSITOL-4-PHOSPHATE IN HUMAN PLATELETS

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Abstract—Non-permeabilizing concentrations (<40 μM) of chlorpromazine (CPZ) increase the radioactivity of phosphatidylinositol-4-phosphate (PIP) in platelets pre-labelled with [^{32}P]P_i, but the biochemical mechanisms underlying this increase are poorly understood. Incubation of [^{32}P]P_i-labelled, gel-filtered platelets with 25 μM CPZ for 10 min increased: (1) the mass of PIP from 315 to 476 nmol/10^{11} platelets but not the total inositol phospholipid mass, (2) the specific phosphodiester radioactivities in phosphatidylinositol (PI), PIP and phosphatidylinositol-4,5-bisphosphate (PIP_2) by 34, 63 and 37%, respectively, and (3) the specific phosphomonoester radioactivities in PIP and PIP_2 by 53 and 10%, respectively. In control platelets (no CPZ) the specific radioactivity of the phosphodiester was the same in PI, PIP and PIP_2, and the specific radioactivity in the phosphomonoester in PIP and PIP_2 was 55% of that of the γ-phosphoryl in ATP, measured as metabolically active, actin-bound ADP. These results suggest that 55% of each of PI, PIP and PIP_2 constitutes a metabolic pool which is labelled by ^{32}P in the platelets, while the remainder is in a metabolically inactive pool and not labelled. CPZ has two major effects: (1) CPZ interferes with the kinase and phosphohydrolase reactions that maintain the steady-state level of PIP in the metabolic phosphoinositide pool, resulting in a 92% increase in the PIP level of this pool, and (2) CPZ causes synthesis (45% in 10 min) of new phosphodiester in the metabolically active phosphoinositides by tentative stimulation of the turnover of the phosphoinositide cycle, *de novo* phosphoinositide synthesis and/or diacylglycerol formation through phospholipases C and D. The marked alteration by CPZ of phosphoinositide metabolism may be part of the mechanism by which this drug effects its psychotropic action.

Phenothiazines are cationic amphiphilic drugs that display general membrane perturbing effects [1, 2] and hence may interfere with cellular processes. However, these drugs permeabilize membranes: at 30 μM, chlorpromazine (CPZ†) lyses artificial lipid bilayer vesicles [3] and above 40 μM phenothiazines cause leakage of low relative molecular mass substances (ATP, glycolytic intermediates) but not high relative molecular mass substances (lactate dehydrogenase) from the platelet cytoplasm [4–7]. Most reported effects of phenothiazines on cellular processes stem from experiments with permeabilizing phenothiazine concentrations (100–3000 μM). It is therefore uncertain whether the effects reflect permeabilization artifacts or specific phenothiazine–membrane interaction.

Reported effects of non-permeabilizing doses of phenothiazines on cellular glycerophospholipid metabolism are confusing. In HeLa cells phosphatidylcholine (PC) biosynthesis was reported to be inhibited by 5 μM CPZ or trifluoperazine (TFP) through specific inhibition of CTP: phosphocholine cytidylyltransferase [8], while in chick heart cells these phenothiazines increased incorporation of [^{3}H]choline into choline phosphate, sphingomyelin (SM) and PC [9]. Furthermore, in GH_3 pituitary cells preincubated with [^{3}H]choline, 25 μM TFP caused degradation of [^{3}H]PC and [^{3}H]SM by activating phospholipase C and SMase [10]. Finally, in platelets 50 μM CPZ was found to stimulate degradation of PC by base exchange with serine [11].

CPZ and TFP (<40 μM) increase the total radioactivity of phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) in platelets prelabelled with [^{32}P]P_i [5, 12–14]. Platelet PIP and PIP_2 have high turnover of their monoester but not of their diester phosphates, and more than 90% of their ^{32}P-radioactivity is confined to the monoester phosphates [15]. The phenothiazine-induced increase of polyphosphoinositide radioactivity could have been caused by increases in mass and/or specific radioactivities of the monophosphoesters and/or the diphosphoesters of the phosphoinositides. The present work investigates these possibilities in order to obtain further insight into the mechanisms of the interaction of phenothiazines with phosphoinositide metabolism.

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† Abbreviations: CPZ, chlorpromazine; GFP, gel-filtered platelets; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; SM, sphingomyelin; TFP, trifluoperazine.

MATERIALS AND METHODS

Chemicals. [^{32}P]P_i (10 mCi/mL, carrier-free, code PBS-11) was from Amersham (U.K.). CPZ (dissolved in 0.9% NaCl and stored in darkness at −20° for less than 1 month) and bovine alkaline phosphatase were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TLC silica gel plates (Art. no. 5553) were from Merck (Darmstadt, Germany). Autoradiography films (Fuji RX medical X-ray) were from Fuji photo film Co. (Tokyo, Japan). Bovine serum albumin (Fraction V) was from Miles Laboratories.

Platelet preparation and labelling. Concentrated platelet-rich plasma obtained from human blood anticoagulated with 11 mM citric acid, 13 mM trisodium citrate and 15 mM dextrose [16] was incubated with [^{32}P]orthophosphate (0.3 mCi/mL) for 60 min at 37°, and gel-filtered into a Ca^{2+}- and phosphate-free Tyrode's solution (pH 7.25–7.3) containing 5 mM glucose and 0.2% (w/v) bovine serum albumin [17]. The gel-filtered platelets (GFP) were diluted with the Tyrode's solution and the final platelet concentration ranged over $2.77–3.5 \times 10^8$ platelets/mL. These prelabelled GFP have the same specific radioactivity of ^{32}P in major metabolites with rapid turnover of their phosphates, i.e. ATP (β- and γ-phosphoryls), ADP (β-phosphoryl), glucose-6-phosphate, fructose-1.6-bisphosphate and P_i [18, 19]. Incubation of such prelabelled platelets in the phosphate-free Tyrode's solution at 37° for at least 1 hr does not cause significant changes in the specific radioactivity of ATP [20] or PIP and PIP_2 [21]. Thus, the [^{32}P]P_i-prelabelled platelets used in this study are in intracellular, metabolic equilibrium with respect to the metabolites with high turnover of their phosphates. PI is not in metabolic equilibrium with these intracellular phosphates, and (about) doubles its specific radioactivity every 30 min during incubation [20].

Incubations. Samples of GFP were equilibrated at 37°, and CPZ solution (25 μM final concentration) or an equal volume of 0.9% NaCl (as control) was added to the suspensions, which were then mixed carefully with gentle tilting. For determination of mass and specific radioactivity duplicate samples were withdrawn after 20 sec and 10 min of incubation. For the time course studies samples were taken at noted times.

Lipid extraction and chromatography. One volume of the incubation mixtures was mixed with 4 vol. of chloroform/methanol/conc. HCl (20:40:1 by vol.) on ice, extracted [19] and separated by TLC using chloroform/methanol/20% methylamine in water (60:36:10 by vol.) [22]. The [^{32}P]phosphoinositide-containing spots were localized by over-night autoradiography and scraped off the plates.

Determination of the specific radioactivity of the diester and monoester phosphates of PI, PIP and PIP_2. The fractions from the second of the duplicate samples were deacylated by monomethylamine/methanol/H_2O/butanol (5:4:3:1) and dephosphorylated by bovine alkaline phosphatase [23]. the [^{32}P]P_i was separated from the remaining glycerophosphateinositol by one-dimensional paper chromatography [15]. The phosphate-containing areas were detected by autoradiography, cut out and the radioactivity was determined by scintillation counting.

Specific radioactivity of actin-bound ADP. About 50% of the cytosolic ADP in human platelets exists as ethanol-insoluble, protein-bound ADP with the same specific radioactivity as other metabolic adenine nucleotides in platelets prelabelled with radioactive adenine [24]. The ethanol-insoluble ADP pool is F-actin-bound ADP [25], and the specific radioactivity of its β-phosphate has been shown to be identical to that of the γ- (and β-) phosphoryl group of metabolic ATP, while the α-phosphate of both ADP and ATP is unlabelled in platelets prelabelled with [^{32}P]P_i [18, 26]. This actin-bound ADP can be separated readily for determination of the specific radioactivity [26]. In short, aliquots of [^{32}P]P_i-labelled GFP (incubated with CPZ or NaCl) were rapidly mixed with 2 vol. of freshly prepared 10 mM EDTA in 86% ethanol, and the F-actin-bound ADP extracted with 0.6 N perchloric acid from the washed ethanol-insoluble fractions. The supernatants were kept for the measurement of ADP mass and radioactivity by high voltage paper electrophoresis and scintillation counting.

Calculations and statistics. Platelets from different donors were used in each of the experiments. Each parameter was assayed in duplicate or triplicate samples taken from the incubation mixture and processed side-by-side. The N-values quoted are therefore the total number of measurements done, i.e. the number of samples that were processed.

The masses of PIP_2, PIP and PI were calculated from the amount of phosphate and adjusted for the different number of phosphate groups. The specific ^{32}P-radioactivity (cpm/nmol) of PI was obtained from the total radioactivity and the total mass of the phosphoinositide. The specific ^{32}P-radioactivities of monoester and diester phosphates of PIP and PIP_2 were calculated from the values obtained for total mass and total ^{32}P-radioactivity of each phosphoinositide and the relative distribution of ^{32}P-radioactivity between the di- and monoester phosphates. Data for PIP_2 were corrected for the presence of two monoester phosphates, assuming that the radioactivity is evenly distributed over the 4- and 5-phosphomonoester groups.

The effect of CPZ on the incorporation of [^{32}P]P_i into the mono- and diester phosphate groups in PIP was compared to the same effects in PIP_2 and PI (diester phosphate only). Measurements in the diester phosphates from PIP_2 and PI were pooled into one group. The differences were statistically explored with maximum likelihood analysis of variance models for incomplete data with two parallel series (within factor) in each series. The analysis was performed by the program 5V in BMDP/PC.

RESULTS

Effect of time of incubation with CPZ on the total radioactivity of platelet phosphoinositides

Incubation of [^{32}P]P_i-prelabelled platelets without CPZ (control platelets) for 30 min did not alter [^{32}P]-PIP_2 or [^{32}P]PIP but caused a 80–120% increase in [^{32}P]PI, as shown previously [20]. In contrast, incubation with 25 μM CPZ caused an increase in [^{32}P]PIP and [^{32}P]PI to 180 and 150, respectively,

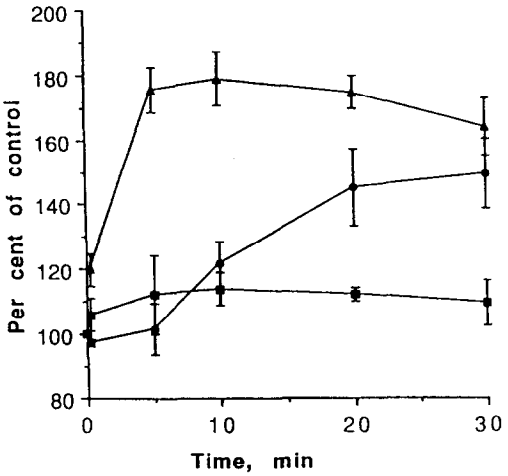

Fig. 1. Effect of CPZ on the time course of incorporation of [^{32}P]P_i and [^{3}H]glycerol into PIP_2, PIP and PI. [^{32}P]P_i-labelled GFP were incubated with or without (control) 25 μM CPZ. The data show the radioactivity in PI (●), PIP (▲) and PIP_2 (■) from a typical experiment representative for platelets from seven different donors, and they are expressed as % of the values obtained from the control samples at each time indicated. For each time point three samples were processed and the curve points show mean ± SD of the corresponding triplicates. Further details about platelet preparation, incubation conditions and determination of radioactivity are described in Materials and Methods. Control platelets at zero time contained 7334, 3295 and 3025 cpm/0.5 mL GFP in PIP_2, PIP and PI, respectively. The radioactivity in PIP_2 and PIP remained constant, while that in PI increased to 4143 cpm/0.5 mL GFP in the controls during the 30 min incubation.

Table 1. CPZ-induced changes in the masses of PI, PIP and PIP_2

		Mass (nmol/10^{11} platelets)	
		20 sec	10 min
PIP_2	Control	231 ± 48 (14)	233 ± 52 (14)
	CPZ	237 ± 43 (15)	261 ± 51 (15)*
PIP	Control	316 ± 67 (12)	315 ± 46 (13)
	CPZ	343 ± 35 (12)	476 ± 53 (13)†
PI	Control	2208 ± 526 (15)	2195 ± 510 (14)
	CPZ	2097 ± 502 (15)	2110 ± 520 (15)*

Gel-filtered platelets were incubated at 37° with (CPZ) or without (Control) 25 μM CPZ. Aliquots of GFP were collected after 20 sec and 10 min of incubation and the mass of each phosphoinositide was determined as described in Materials and Methods.

Data represent means ± SD from seven different experiments. The number of determinations is given in parenthesis.

* CPZ vs control: not significant.

† CPZ vs control: significant, $P < 0.005$.

Table 2. Specific radioactivity of the monoester phosphates of PIP_2 and PIP and the metabolic pool of ATP monitored as the specific radioactivity of actin-bound ADP in control platelets

Time	Actin-bound ADP	Monoester phosphate	
		PIP_2	PIP
20 sec	100	52 ± 15	55 ± 13
10 min	100	58 ± 23	52 ± 17

[^{32}P]P_i-labelled GFP was incubated for 20 sec and 10 min at 37°, and the specific radioactivity of actin-bound ADP and the monoester phosphates was determined as described in the text. The specific radioactivity of actin-bound ADP in the control samples is defined as 100%.

The data are from experiments with blood from eight donors and are means ± SD.

relative to the radioactivity (100%) of each phospholipid in the control platelets (Fig. 1). There was no significant change in [^{32}P]PIP_2. [^{32}P]PIP peaked after 5–10 min and then declined slowly, while [^{32}P]PI increased throughout the whole of the 30 min incubation but mostly between 5 and 20 min. CPZ did not cause incorporation of ^{32}P into other platelet phospholipids (data not shown).

Effect of CPZ on the mass of phosphoinositides

From the time course experiments (Fig. 1) it was clear that the increase in [^{32}P]PIP reached a maximum after 5–10 min of incubation of the platelets with CPZ. Our further studies on mass and specific radioactivities were therefore performed with an incubation time of 10 min. Within this time period 25 μM CPZ caused a 12 and 51% increase in the mass of PIP_2 and PIP, respectively, but a 15% reduction in the mass of PI (Table 1). However, only the increase in the mass of PIP was significant ($P < 0.0019$). The CPZ-induced increase in the masses of PIP_2 and PIP was slightly greater than the reduction in PI, resulting in a net increase in the total phosphoinositide mass of about 100 nmol/10^{11} platelets (3.8%). This increase in mass was not however significant and the total amount of phosphoinositide therefore may be unaffected by the addition of CPZ.

Specific ^{32}P-radioactivity of the di- and monoester phosphates of the phosphoinositides and of the γ-phosphoryl group of ATP before and after incubation with CPZ

Control platelets. The specific ^{32}P-radioactivity of PIP_2 and PIP monoester phosphates in the control platelets was only 58 and 52%, respectively, of the specific radioactivity in actin-bound ADP (Table 2). This result is in contrast to our earlier observations and indicates that there are metabolically heterogenous pools of PIP and PIP_2 in human platelets (see Discussion and Fig. 2). The specific radioactivity of the diester phosphates was statistically the same in PI, PIP and PIP_2 from control platelets (Table 3), but only 2–6% of that of the γ-phosphoryl group

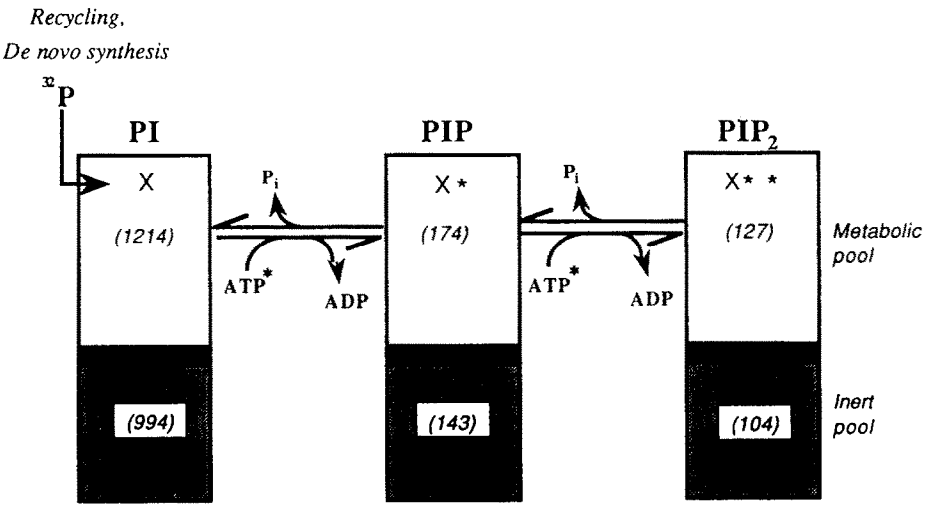

Fig. 2. Possible model for metabolic compartmentalization of inositol phospholipids in resting platelets which takes into account that the phosphodiesters are in mutual metabolic equilibrium while the phosphomonoesters are not in equilibrium with the γ-phosphoryl of ATP. Radiolabelling is designated X for [^{32}P]phosphodiester and * for [^{32}P]phosphomonoester and γ-[^{32}P]phosphoryl in ATP. The number in parenthesis shows the pool size, expressed as nmol/10^{11} platelets. Fifty-five per cent of each of PI, PIP and PIP_2 is present in a compartment where the phosphomonoesters of PIP and PIP_2 are in metabolic equilibrium with the γ-phosphoryl of ATP, and the phosphodiesters of all three inositol phospholipids are in equilibrium with each other (but not with ATP). The remaining 45% of each of PI, PIP and PIP_2 are present in a non-metabolic compartment where both phosphomonoesters and phosphodiesters are not accessible for ^{32}P-radioactivity from ATP.

Table 3. Normalized specific ^{32}P-radioactivity (SPR) of the diester phosphate groups in PI, PIP and PIP_2 in control platelets

	PI	PIP	PIP_2
Range of SRA	438–1277	344–1307	299–1723
Normalized	100	100.25 ± 23*	111 ± 52*

[^{32}P]P_i-prelabelled GFP were incubated with 0.9% NaCl for 10 min and the relative specific radioactivity of the diester phosphate group was determined as described in the text (N = 8).

* Not significantly different from PI.

Table 4. The effect of 25 μM CPZ on the [^{32}P]P_i incorporation into the different phosphate groups of PI, PIP and PIP_2

	20 sec	10 min
PIP_2	D 103 ± 12 (9)	134 ± 14 (9)*
	M 104 ± 7 (7)	110 ± 8 (9)†
PIP	D 109 ± 22 (8)	163 ± 36 (11)*
	M 115 ± 19 (10)	153 ± 17 (9)*
PI	D 105 ± 13 (11)	137 ± 21 (14)*

Samples of [^{32}P]P_i-labelled GFP were incubated at 37° with or without 25 μM CPZ for 20 sec or 10 min. The specific ^{32}P-radioactivity of the monoester and diester phosphates of each phosphoinositide was determined as described in Materials and Methods.

The results are presented as % of control and are means ± SD from six separate experiments, and represent relative specific radioactivities. The number of different determinations is indicated in parenthesis.

M, monoesterphosphate; D, diesterphosphate.

Significance: 10 min vs 20 sec. * Significant, $P < 0.005$; † not significant, $P > 0.1$.

in ATP (data not shown). We found in this study, as observed for PI previously [20], that the specific radioactivity of the phosphodiester increased with time after terminating the [^{32}P]P_i pulse, i.e. gel filtration of the platelets.

CPZ-treated platelets. Incubation of platelets for 10 min with CPZ did not alter the specific ^{32}P-radioactivity of actin-bound ADP (data not shown) and hence did not alter that of metabolically active ATP. The specific radioactivity of the diester phosphate in PI increased by 37% during the 10 min incubation with CPZ (Table 4), which correlates well with the increase in total ^{32}P-radioactivity of PI (Fig. 1A). A corresponding CPZ-induced increase of 34% in the specific radioactivity in the diester phosphate of PIP_2 was seen, but no significant change in the specific radioactivity of the PIP_2 monoester phosphates (Table 4). On the other hand, a totally different profile of the incorporation of [^{32}P]P_i into the phosphate groups was found for PIP in that CPZ increased the specific radioactivity of the phosphodiester and phosphomonoester by 63 and 53%, respectively (Table 4).

That CPZ exerts a greater effect of ^{32}P incorporation into PIP compared to PI and PIP_2 was

Table 5. Specific radioactivities: statistical analysis of the effect of CPZ on PIP compared to PIP_2 and PI using MC-analysis of variance model

	Factor	χ^2	df	P
(a)	PIP vs PIP_2/PI	13.51	1	<0.001
(b)	M/D	3.72	1	<0.054
(c)	Interaction between (a) and (b)	9.80	1	<0.002

The analysis was performed as described in Materials and Methods.

(a) Demonstrates the significantly more profound effect of CPZ on PIP than on PIP_2 and PI.

(b) The increase in specific ^{32}P-radioactivity caused by CPZ is not significantly different between the total mono- and diester phosphate groups of PI, PIP and PIP_2.

(c) The interaction between (a) and (b). The CPZ effect on PIP compared to PIP_2 and PI is significantly more profound in the monoester phosphate groups than in the diester phosphate groups.

clearly significant when the di- and monoester phosphate measurements were combined in an analysis of variance ($P < 0.001$, Table 5a). Furthermore, this effect was greater in the PIP monoester phosphate than in the PIP diester phosphate ($P < 0.002$, Table 5c).

DISCUSSION

The specific radioactivity of the phosphomonoesters of PIP_2 and PIP was 55% of that in the γ-phosphoryl group of ATP. This is in contrast to our previous studies where these specific radioactivities were regarded as equal, suggesting metabolic equilibrium between ATP and inositide monoesters [15, 21]. There are three apparent reasons for the discrepancy: (1) the method for determination of the specific radioactivity of ATP used previously gave values that are 19% lower than those given by the method used here, for reasons discussed elsewhere [25]. (2) In the former studies the specific radioactivities were not statistically different, but their mean values for the phosphomonoester were 10% lower than that of ATP. (3) The masses are, for unknown reasons, higher in the present than in previous studies. The present values are based on more measurements (seven donors, 28 determinations) than the previous studies (three donors, eight determinations) and are statistically more reliable.

A specific phosphomonoester radioactivity in PIP and PIP_2 that is 55% lower than that of the γ-phosphoryl group of ATP indicates metabolic heterogeneity of the two inositides in human platelets. Since the specific radioactivities of the phosphodiesters of PI, PIP and PIP_2 were practically the same (Table 3, [21]), the simplest (and most plausible) explanation of these specific radioactivity data is that *55% and 45% of each of the three inositides is in a metabolic and a non-metabolic pool, respectively*. This model is depicted in Fig. 2, and is further supported by the following observation: after removal of precursor $[^{32}P]P_i$ from platelets, the specific radioactivity of the phosphodiesters is 3–4% of that in the γ-phosphoryl of ATP and increases with further incubation (to 20% of the γ-phosphoryl of ATP after 90 min), while the specific monoester radioactivity does not change significantly [20, 21], remaining at 55% of that in the γ-phosphoryl of ATP.

Metabolic heterogeneity of phosphoinositides has been suggested in rabbit platelets [27], human erythrocytes [28, 29] and mouse fibroblasts [30]. The metabolic and non-metabolic pools of inositide in platelets (Fig. 2) may be present in different membrane systems: $[^{32}P]P_i$-labelled PI, PIP and PIP_2 are present in the plasma membrane exclusively; i.e. not in internal membranes [31] or the granule membranes (Horvli and Holmsen, unpublished observation). The internal membranes contain considerable amounts of PI [32], so that the metabolic and non-metabolic pools in Fig. 2 may be located in plasma and internal membranes, respectively. Furthermore, PIP and PIP_2, but not PI, are known to be strongly associated with cytoskeletal proteins [33–37], protein kinase C [38–40] as well as plasma membrane Ca^{2+}-transport ATPase [41]; such protein-bound phosphoinositides may be metabolically inert. It is also possible that the metabolic heterogeneity of the inositol phospholipids stems from inter-platelet differences which are further discussed below.

One major effect of non-permeabilizing CPZ was a parallel increase in the mass and total radioactivity of PIP, the latter being confined mainly to its phosphomonoester. This suggests that CPZ causes changes in the relative rates of the two kinase and phosphomonoesterase reactions that maintain the level of PIP resulting in elevation of the steady-state level of PIP, as depicted in Fig. 3. The finding that CPZ (<50 μM) slightly stimulates the PI phosphorylation [42] and powerfully inhibits the PIP phosphorylation [43] by adrenal medulla granules *in vitro* is therefore interesting, and we are presently studying the effects of CPZ on these enzymes from platelets. The PIP isomer PI-3-phosphate constituted less than 1% of the PIP synthesized during the CPZ action on platelets (Miriam Fukami and Holm Holmsen, unpublished results), showing that CPZ only acted on 4- and 5-kinases and phosphatases.

The specific radioactivity, relative to that of the γ-phosphoryl in ATP, of the phospho*mono*ester in the PIP synthesized during the CPZ–platelet interaction was 39% higher than that of the γ-phosphoryl group of ATP (relative specific radioactivity, Table 6). This shows that the increase in the steady-state level of PIP is confined to the metabolic pool. Since the average increment in the mass of PIP is 161 nmol/10^{11} platelets (Table 6) and the size of the metabolic PIP pool is 174 nmol/10^{11} platelets, 10 min of CPZ treatment increases this pool by 92%. Furthermore, the higher specific radioactivity in the PIP phosphomonoester than in the γ-phosphoryl of ATP shows that synthesis of PIP occurs in compartments where ATP has a higher specific radioactivity than its *mean* specific radioactivity, which we have measured. It is hard to conceive of pools of ATP with different turnovers within the metabolic compartment of *one* platelet

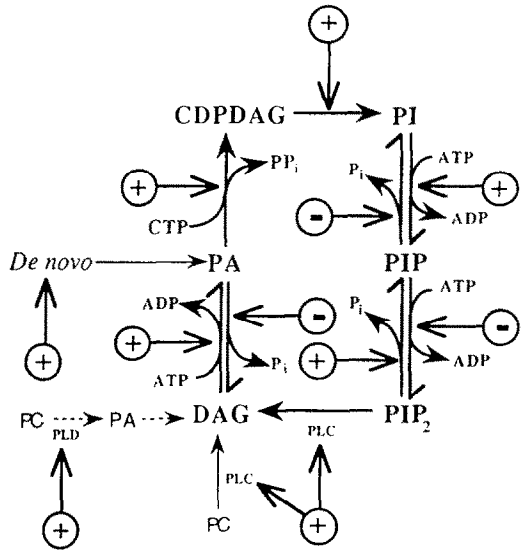

Fig. 3. Possible sites of action of CPZ on platelet phospholipid metabolism. CPZ is thought to act in the metabolic compartment (Fig. 2); arrows marked with encircled + (stimulation) or − (inhibition) suggest specific reactions affected by CPZ. The inositol phospholipid cycle is shown in bold face since the CPZ-promoted changes in the rate of the reactions in this pathway represent the most plausible explanation for synthesis of 46% more phosphodiester without increase in total mass, and a 51% increase in the steady-state level of PIP. See text for further explanation.

Table 6. Calculated specific and total radioactivities of the monoester phosphates in PIP and PIP_2 formed after 10 min of platelet–CPZ interaction relative to the specific radioactivity of the γ-phosphoryl in ATP

	Mass	RSRA	RTRA
PIP			
Control	*315*	*54.24*	17,085
CPZ	*476*	83.0*	39,501
ΔPIP	161	**139.2**	22,417
PIP_2			
Control	*233*	*54.24*	12,637
CPZ	*261*	59.7†	15,581
ΔPIP_2	28	**105.1**	2944

The data for mass in italics are not taken from Table 1 (10 min). The relative specific radioactivity (RSRA) in the monoester phosphates of PIP and PIP_2 in control platelets is the average of the values in Table 2 (52.24) while RSRA after CPZ treatment is calculated as indicated. The relative *total* radioactivity (RTRA) has been calculated as mass × RSRA. The increment in mass and RTRA during incubation with CPZ has been calculated (Δ) as $mass_{CPZ} - mass_{control}$ and $RTRA_{CPZ} - RTRA_{control}$, respectively. The RSRA of the phosphoester formed after the CPZ action has been calculated as ΔRTRA/Δmass and is shown in bold face.

* 54.24 × 153/100, where 153 is RSRA of PIP monophosphoester at 10 min (Table 4).

† 54.24 × 110/100, where 110 is RSRA of PIP_2 monophosphoester at 10 min (Table 4).

Table 7. Calculated relative specific and total radioactivities of the diester phosphates of polyphosphoinositides formed after 10 min of platelet–CPZ interaction as related to the specific radioactivity of PI in control cells

	Mass	RSRA	RTRA
PI			
Control	*2195*	*100*	219,500
CPZ	*2110*	*137*	289,020
ΔPI	−85	—	69,520
PIP			
Control	*315*	*100*	31,578
CPZ	*476*	*163*	77,588
ΔPIP	161	**286**	46,009
PIP_2			
Control	*233*	*111*	25,863
CPZ	*261*	*134*	34,974
ΔPIP_2	28	**325**	9111
PPI*			
Control	2743	—	276,941
ΔPPI	103	—	124,640

The data for mass in italics are taken directly from Table 1 (10 min) while the relative specific radioactivity data in italics for control are from Table 3 and those for CPZ are from Table 4 (diester at 10 min). Further calculations and expressions are as in Table 6.

* PPI = PI + PIP + PIP_2.

[44], but *different platelets* may have different ATP turnovers since platelet sub-populations that can be separated by size, shape and weight are metabolically different [45–47].

The other major effect of non-permeabilizing CPZ was a marked increase in the specific radioactivity of the phospho*di*ester of all three phosphoinositides. This means that CPZ caused synthesis of *new* diester, and Table 7 shows that incubation of platelets with CPZ for 10 min gives 45% new phosphodiester (in PI + PIP + PIP_2), with a percentage distribution of 54.6, 36.2 and 9.1 in PI, PIP and PIP_2, respectively. Since the new phosphodiester is synthesized in the metabolic pool containing 1.515 nmol/10^{11} cells of PI + PIP + PIP_2 (Fig. 2), incubation of platelets with CPZ yields 68.1 nmol/10^{11} cells in 10 min, thus with an average rate or 68.1 nmol/min/10^{11} cells. Irrespective of its mechanism of formation, the phosphodiester (see below) will appear in PA before it is incorporated into the inositol phospholipids. No PA accumulation was, however, seen during the platelet–CPZ interaction. This makes sense since the rate of PA removal in resting platelets is about 52 nmol/min/10^{11} cells [48], thus close to the rate of CPZ-induced phosphodiester formation.

Although it is clear that CPZ causes the synthesis of new phosphoinositide molecules, the present results do not distinguish between the several possible mechanisms (Fig. 3) that could lead to this synthesis. These mechanisms are now being studied by us and we are initially attempting to determine how the phosphodiester is formed, as follows.

Increase in phosphoinositide cycling. The 3.7% increase in total mass of phosphoinositides (Table 7) was not significant, and phosphodiester formation could occur without increase in total phosphoinositide

mass. CPZ may increase the turnover rate of the phosphoinositide cycle (Fig. 3, bold face) by interacting with one or several steps as shown in Fig. 3.

De novo synthesis. The newly synthesized phosphoinositides are in the metabolic pool, the mass of which may increase significantly, despite the insignificant increase in the combined mass of both phosphoinositide pools. In permeabilizing concentrations CPZ redirects the *de novo* synthesis of glycerophospholipids in liver cells from formation of neutral to acidic lipids conceivably through inhibition of PA phosphohydrolase [49–51]. At 25 μM, CPZ gave most of the indications of redirection as at permeabilizing concentrations, albeit to a lesser degree [52]. TFP (<40 μM) inhibits PA phosphohydrolase from platelets [53], and it is possible that the increase in phosphodiester formation by CPZ is due to redirection.

Diesteratic hydrolysis of PC and PE. The phosphodiester could also be formed in the diacyclycerol kinase reaction from diacylglycerol originating from PC or PE through hydrolysis with phospholipase C or D (after hydrolysis of PA) with CPZ acting as shown in Fig. 3. The PA originating directly from PE or PC in platelets by a putative CPZ activation of phospholipase D cannot contain the synthesized phosphodiester, since PC and PE are not labelled in platelets by [^{32}P]P_i [53] while the phosphodiester synthesized by CPZ action is labelled.

Several receptor-controlled functions in nervous tissue utilize the phosphoinositide signal transduction system [54]. The profound effect of low concentrations of CPZ on the metabolism of phosphoinositides shown here may be part of the mechanism by which this drug effects its psychotropic action.

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