Differential effects of chlorpromazine on secretion, protein phosphorylation and phosphoinositide metabolism in stimulated platelets

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1. Increasing concentrations of chlorpromazine (30–500 μM) caused a progressive lysis of gel-filtered platelets, as monitored by the extracellular appearance of cytoplasmic (^{14}C)adenine-labelled) adenine nucleotides. The chlorpromazine-induced lysis was markedly enhanced by thrombin and phorbol ester, and complete cytolysis was found at chlorpromazine concentrations of 100 μM and above in the presence of thrombin. 2. At non-lytic concentrations, chlorpromazine caused a dramatic increase in the thrombin- or phorbol ester-mediated incorporation of ^{32}P into phosphatidylinositol 4-phosphate and, to a lesser extent, into phosphatidylinositol 4,5-bisphosphate in platelets pulse-labelled with [^{32}P]P_i. Chlorpromazine alone also caused an incorporation of ^{32}P into the phosphoinositides. 3. Non-lytic concentrations of chlorpromazine had no effect on the phosphorylation of the 47 kDa protein (regarded as the substrate for protein kinase C), but markedly inhibited the accompanying secretion of ATP + ADP and β -hexosaminidase when platelets were incubated with 0.17 μM -phorbol ester or 0.1–0.2 unit of thrombin/ml. At lower concentrations of thrombin, chlorpromazine did not inhibit, but slightly enhanced, secretion. 4. A protein of 82 kDa was phosphorylated during the interaction of platelets with thrombin and phorbol ester, and this phosphorylation was enhanced by chlorpromazine (non-lytic). 5. These results suggest that the previously reported inhibition of protein kinase C by chlorpromazine is probably non-specific and due to cytolysis. However, since non-lytic concentrations of chlorpromazine inhibit secretion, but not protein kinase C, in platelets, activation of protein kinase C is not involved in the stimulation–secretion coupling, or chlorpromazine acts at a step after kinase activation. Possible mechanisms of this inhibition by chlorpromazine are discussed in the light of its effect on phosphoinositide metabolism and protein phosphorylation.

INTRODUCTION

It is well established that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) activates platelet protein kinase C, as monitored by phosphorylation of a 47 kDa protein (P47) (Castagna *et al.*, 1982; Tapley & Murray, 1984). TPA has also been shown to stimulate dense-granule secretion in platelets without an increase in cytoplasmic Ca^{2+} (Rink *et al.*, 1983; Sanchez *et al.*, 1983); this stimulation is believed to be mediated by phosphorylation of P47 alone and not by Ca^{2+} mobilization. TPA was shown to cause an increase in the ^{32}P content of the polyphosphoinositides of platelets pulse-labelled with [^{32}P]P_i, thought to be due to an interference with the kinases and phosphatases maintaining the steady-state concentrations of the polyphosphoinositides (de Chaffoy de Courcelles *et al.*, 1984a; Halenda & Feinstein, 1984).

Nishizuka & Takai (1981) have reported that chlorpromazine inhibits the thrombin-induced phosphorylation of P47 in washed platelets pulse-labelled with [^{32}P]P_i. Chlorpromazine is an amphiphilic cationic drug which interferes with the organization of biological membranes (Bowley *et al.*, 1977). In humans the toxic doses of this drug in plasma is 1 $\mu\text{g}/\text{ml}$ or 2.8 μM (Gilman *et al.*, 1980). Therefore the chlorpromazine concentration of 500 μM used in platelet studies (Nishizuka & Takai, 1981) and in studies with other cells (Sturton & Brindley, 1977;

Shoyab *et al.*, 1982; Zborowski & Brindley, 1983) must be considered extremely high and perhaps of little relevance under conditions *in vivo*. In fact, we show in the present paper that even substantially lower concentrations of chlorpromazine are cytolytic. Using the highest non-lytic concentration of chlorpromazine, we have re-investigated its effect on TPA- and thrombin-mediated stimulation of polyphosphoinositide metabolism, protein phosphorylation and secretion in human platelets.

MATERIALS AND METHODS

Blood from normal human donors who denied having taken any medication for at least 10 days was collected into 0.1 vol. of 0.15 M-sodium citrate and centrifuged at 190 g_{max} in a Heraeus Christ Minifuge GL for 15 min at 24 °C for preparation of platelet-rich plasma (PRP). Platelet counts were determined with a Coulter Counter ZM (Coulter Electronics) and adjusted to 3.5×10^8 cells/ml in the suspensions used for all types of incubation experiments with chlorpromazine.

Platelet lysis

Gel-filtered human platelets (GFP) prepared as described by Lages *et al.* (1977) from PRP preincubated for 60 min with 0.8 μM -[U- ^{14}C]adenine (276 mCi/mmol;

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PRP, platelet-rich plasma; GFP, gel-filtered platelets; PI, PIP, PIP₂, phosphatidylinositol, its 4-phosphate and 4,5-bisphosphate; PA, phosphatidic acid.

Amersham; CFA 436) were obtained in Ca^{2+} -free Tyrode's solution containing 0.2% bovine serum albumin (Miles Laboratories) and 5 mM-glucose. The ^{14}C -labelled adenine nucleotides are exclusively present in the extragranular compartments (Holmsen *et al.*, 1974; Ugurbil *et al.*, 1979) inside the platelets, and their appearance extracellularly is a measure of cell lysis (Lages *et al.*, 1983). Samples of the pulse-labelled GFP (2×10^8 – 3×10^8 cells/ml) were incubated at 37 °C with bovine thrombin (0.2 unit/ml; Topical, La Roche), TPA (0.17 μM ; P-L Biochemicals) or NaCl (0.15 M) for 1 min. Various concentrations of chlorpromazine (Sigma) were added 2 min before these incubations. To measure lysis, 0.3 ml samples were transferred into precooled Eppendorf tubes on ice containing 50 μl of 1 M-formaldehyde/50 mM-EDTA to prevent further lysis or secretion (Holmsen & Setkowsky-Dangelmaier, 1977; Holmsen & Dangelmaier, 1981). The tubes were centrifuged for 2 min at 12000 g , and 200 μl portions of the supernatants (test supernat.) were mixed with 200 μl of EDTA/ethanol (Holmsen *et al.*, 1984a) on ice for nucleotide extraction. Similar extracts were prepared from non-centrifuged GFP that had not been incubated with thrombin or TPA (total) and from the supernatant from this GFP (control supernat.). The nucleotides in these EDTA/ethanol extracts were separated by high-voltage paper electrophoresis, and their ^{14}C content was determined as described by Holmsen *et al.* (1974). The percentage lysis was calculated from the sum of the radioactivities (R) of ATP, ADP, AMP and IMP:

$$\% \text{ lysis} = 100 \times \frac{R(\text{test supernat.}) - R(\text{control supernat.})}{R(\text{total}) - R(\text{control supernat.})}$$

Incubations for studies of phosphoinositide metabolism, protein phosphorylation and secretion

PRP was incubated at 37 °C for 60 min with 0.2 mCi of [^{32}P]P_i/ml (carrier-free; Amersham; PBS 11) and gel-filtered as described above, except that the Tyrode's solution did not contain added P_i. Samples of such pre-labelled GFP were incubated without stirring at 37 °C with thrombin (in various concentrations), TPA (0.17 μM), NaCl (0.15 M), chlorpromazine (20 or 30 μM) and with chlorpromazine plus thrombin or TPA. Chlorpromazine was added 2 min before stimulation with thrombin and TPA. Samples were taken at the times noted for phospholipid extractions, protein phosphorylation and secretion.

For studies of phosphoinositide metabolism, the samples (0.5 ml) were each mixed with 2 ml of chloroform/methanol/HCl (20:40:1, by vol.) on ice. Water (1 ml) and chloroform (1 ml) were added with vigorous shaking, and the resulting mixtures were centrifuged for 10 min at approx. 200 g (room temperature) to enhance phase separation. The bottom phase was collected, evaporated and dissolved in chloroform/methanol (2:1, v/v). Separation of PI, PIP, PIP₂, PA and other phospholipids were performed on aluminium sheets coated with silica gel 60 (Merck; DC Alufolien) developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) as the mobile phase (Imai *et al.*, 1983) or chloroform/methanol/20% methylamine (12:7:2, by vol.) (de Chaffoy de Courcelles *et al.*, 1984a). The spots were localized by autoradiography;

they were then cut out and their content of ^{32}P was measured by liquid-scintillation counting.

Protein phosphorylation was studied in two ways. (a) To determine the various proteins that were phosphorylated during the incubation, 100 μl samples of the incubation mixture were diluted with 50 μl of electrophoresis buffer containing 6.3% (w/v) SDS, 150 mM-Tris/HCl, pH 6.7, 15.2% (w/v) glycerol and 33.3% (v/v) 2-mercaptoethanol. These samples were heated for 3 min at 95 °C. One-dimensional polyacrylamide (9%) gel electrophoresis in SDS was performed for 1230 V-h to separate the proteins in a slab-gel system (Laemmli, 1970). The gels were stained with Coomassie Blue, dried and subjected to autoradiography. After the films were developed, the protein spectra were analysed with a scanning densitometer (Zenoh). (b) For determination of the phosphorylation of the 47 kDa substrate of protein kinase C (P47), 0.5 ml samples of the incubation mixture were mixed with 0.5 ml of ice-cold 0.6 M-HClO₄. The protein sediments were pelleted by centrifugation for 2 min at 12000 g (room temperature) and washed once with distilled water. The pellets were kept at –20 °C overnight and then dissolved by heating at 95 °C (Eppendorf Thermoblock) for 3–4 min in 45 μl (per 10⁸ platelets) of sample buffer (11% glycerol, 113 mM-Tris base, 2.3% SDS, 30 mM-dithiothreitol). Portions (50 μl) of these solutions were electrophoresed on 13.6% polyacrylamide gel with 150 mM-Tris/HCl (pH 8.8) 0.4% SDS, at 10 mA overnight. The radioactive bands, observed by autoradiography, were cut out and the radioactivity was determined by scintillation counting. The variations in the ^{32}P content of P47 are expressed by relative values, i.e. P47 radioactivity/fraction A radioactivity, where fraction A is a phosphoprotein labelled by ^{32}P in unstimulated platelets and which does not change its radioactivity during stimulation (see Fig. 3).

Samples for determination of secreted substances were prepared during incubation as described previously (Holmsen *et al.*, 1984b). Dense-granule secretion was determined as secreted ATP+ADP (Steen & Holmsen, 1985), and acid hydrolase secretion was determined as secreted β -hexosaminidase (Dangelmaier & Holmsen, 1980).

RESULTS

Platelet lysis

Fig. 1 shows that GFP stimulated by thrombin (0.2 unit/ml) or TPA (0.17 μM) were not lysed in the presence of chlorpromazine in concentrations below 30 μM . However, increasing chlorpromazine concentrations from 20 to 500 μM resulted in a progressive cytolysis, and complete lysis was achieved at 100 μM and higher concentrations of the drug. At these elevated drug concentrations even chlorpromazine alone caused considerable cytolysis, although TPA and thrombin, in particular, enhanced the chlorpromazine-induced lysis. Thus with GFP the highest non-lytic concentration of chlorpromazine is 20–30 μM .

Phosphoinositide metabolism

Similarly to previous reports (de Chaffoy de Courcelles *et al.*, 1984a; Halenda & Feinstein, 1984), TPA caused a marked increase in [^{32}P]PIP, but little change in [^{32}P]PIP₂ within 60 s of incubation with the ^{32}P -labelled

Fig. 1. Effect of chlorpromazine on lysis of human platelets

Gel-filtered [^{14}C]adenine-labelled platelets were preincubated for 2 min with the indicated amount of chlorpromazine and then further incubated for 2 min with 0.2 unit of thrombin/ml (■), 0.17 μM -TPA (●) or 0.15 M-NaCl (○). All incubations were performed at 37 °C. Percentage lysis was calculated from the amount of ^{14}C -labelled adenine nucleotides determined extracellularly as described in the Materials and methods section. The results shown are mean values of three different experiments.

GFP (Figs. 2a and 2b). TPA caused no significant change in [^{32}P]PI (Fig. 2c) and no formation of [^{32}P]phosphatidate (PA) (results not shown). Chlorpromazine alone induced a more rapid increase in [^{32}P]PIP than did TPA (Fig. 2b), and also caused a 30% increase in the ^{32}P content of PIP_2 (Fig. 2a) and a decrease in [^{32}P]PI (Fig. 2c). When present together, TPA and chlorpromazine caused almost a doubling of [^{32}P]PIP $_2$ within 20 s of incubation (Fig. 2a), whereas the increase in [^{32}P]PIP was about the same as with chlorpromazine alone (Fig. 2b); [^{32}P]PI decreased, although not as much as with chlorpromazine alone (Fig. 2c).

It is well established that thrombin induces a transient decrease followed by a rapid thrombin-induced increase in [^{32}P]PIP $_2$, an increase in the ^{32}P content of PIP and a steady increase in [^{32}P]PI (for references, see Holmsen *et al.*, 1984b). These thrombin-induced changes are shown in Figs. 2(a) and 2(c), which also demonstrate that the presence of chlorpromazine enhanced the increase in [^{32}P]PIP $_2$ (Fig. 2a) and particularly in [^{32}P]PIP (Fig. 2b), but did not alter the thrombin-induced increase in [^{32}P]PI (Fig. 2c) or in [^{32}P]PA (results not shown).

Fig. 2. Effect of TPA and thrombin in the presence or absence of chlorpromazine on the ^{32}P content of phosphoinositides

Gel-filtered [^{32}P]P $_i$ -labelled platelets were incubated in the presence or absence of chlorpromazine (20 μM) or NaCl (0.15 M) with 0.2 unit of thrombin/ml (■, thrombin alone; □, thrombin + chlorpromazine), 0.17 μM -TPA (●, TPA alone; ○, TPA + chlorpromazine) or 0.15 M-NaCl (△, chlorpromazine alone). Incubation without chlorpromazine gave negligible changes (results not shown). The reactions were terminated after the indicated times by

the addition of chloroform/methanol/HCl (20:40:1, by vol.), and the radioactivity of PIP $_2$ (a), PIP (b) and PI (c) was determined as described in the Materials and methods section.

Fig. 3. Densitometric scan of autoradiographs of [32 P]phosphorylated proteins in platelets treated with thrombin and TPA in the presence and absence of chlorpromazine

Gel-filtered platelets, pulse-labelled with [32 P] P_i , were incubated for 2 min with 0.2 unit of thrombin/ml, 0.17 μ M-TPA and/or 20 μ M-chlorpromazine in the combinations shown for each scan. For further details, see the Materials and methods section, protein phosphorylation method (a). Equal amounts of protein from each incubation were electrophoresed; molecular masses are indicated with arrows. (The strongly radioactive fraction A did not change its 32 P content under any treatment and is used as an internal reference in Figs. 4, 5 and 6). In this particular scan, proteins with molecular masses lower than 25 kDa are not present because they run off the gel.

Protein phosphorylation

It has previously been reported that phosphorylation of the platelet protein P47 was inhibited by 500 μ M-chlorpromazine (Nishizuka & Takai, 1981; Shoyab *et al.*, 1982). In view of our present finding that this concentration of chlorpromazine causes complete lysis of stimulated platelets, we re-investigated the effect of chlorpromazine at a non-lytic concentration (20 μ M) on protein phosphorylation induced by TPA and thrombin in intact platelets. No significant inhibition of P47 phosphorylation was observed (Fig. 3). The lack of effect was not related to type of agonist, since both thrombin (0.2 unit/ml) and TPA (0.17 μ M) produced a similar degree of phosphorylation. Chlorpromazine (20 μ M) alone had no effect on P47 phosphorylation.

However, TPA and thrombin caused phosphorylation of three other proteins, one of 67 kDa (P67) and two of 80–90 kDa. The 82 kDa protein (P82) was phosphorylated in non-stimulated platelets to a small extent. Phosphorylated proteins of 60 and 82 kDa are

present on gels from stimulated platelets published by Haslam *et al.* (1980), although they were not commented on. In the present study treatment of the platelets with chlorpromazine, TPA or thrombin caused a reproducible increase in P82 phosphorylation (Fig. 3). An additive effect on the phosphorylation of the 80–90 kDa proteins was found when the platelets were treated with combinations of chlorpromazine and TPA or chlorpromazine and thrombin. Phosphorylation of P67 was not observed in controls or in chlorpromazine-treated GFP. Both thrombin and TPA stimulated such a phosphorylation, but chlorpromazine (20 μ M) had no effect on phosphorylation of P67, thus in contrast with the effect of chlorpromazine on P82.

Effect of a non-lytic concentration of chlorpromazine on platelet secretion and phosphorylation of P47 in intact platelets

Sanchez *et al.* (1983) reported that chlorpromazine at 30 μ M, a non-lytic concentration, abolished secretion of ATP from washed platelets induced by 20 μ M-TPA in the presence of 1 mM extracellular Ca^{2+} . In our GFP, which contained 32 P-labelled platelets with no added Ca^{2+} , TPA caused a slow secretion of ATP+ADP after a lag of 1–2 min (Fig. 4c). Chlorpromazine (30 μ M) produced a 50–60% inhibition of this secretion (Fig. 4c). However, TPA also induced a slow secretion of β -hexosaminidase, which started after a lag, and this response was abolished by 30 μ M-chlorpromazine (Fig. 4b). Phosphorylation of P47 started immediately on addition of TPA and reached a maximum after about 2 min, after which the 32 P content of P47 decreased slowly (Fig. 4a). Chlorpromazine had no effect on the initial increase in 32 P-labelled P47, but the phosphorylation reached a plateau at a lower value than in the absence of chlorpromazine and did not decrease; after 16 min of incubation, the extent of phosphorylation was the same in the presence of chlorpromazine as in its absence (Fig. 4a). Thus these time-course studies revealed a substantially smaller inhibitory effect of chlorpromazine on TPA-induced phosphorylation than on the secretory responses, particularly for β -hexosaminidase secretion.

The effect of 30 μ M-chlorpromazine on P47 phosphorylation, β -hexosaminidase secretion and ATP+ADP secretion during 30 s incubation of platelets with increasing concentrations of thrombin is shown in Fig. 5. The degree of P47 phosphorylation did not vary with the concentration of thrombin up to 0.2 unit/ml, and was only slightly inhibited by chlorpromazine at 0.1 unit/ml and lower thrombin concentrations (Fig. 5a). In contrast, secretion of both β -hexosaminidase (Fig. 5b) and ATP+ADP (Fig. 5c) was actually potentiated by chlorpromazine below 0.1 unit/ml and progressively inhibited as the thrombin concentration increased from 0.1 to 0.2 unit/ml. Since both the secretory responses were 60–70% inhibited by chlorpromazine at 0.2 unit of thrombin/ml, the effects of the drug on the time course of these responses were studied at this thrombin concentration (Fig. 6). Thrombin caused a very rapid increase in P47 phosphorylation, and chlorpromazine had a slight, transient, inhibitory effect (Fig. 6a). In contrast, chlorpromazine markedly inhibited β -hexosaminidase secretion (Fig. 6b) and caused a 50% inhibition of the secretion of ATP+ADP (Fig. 6c). Thus the non-lytic concentration of chlorpromazine markedly inhibited the progress of the thrombin-induced secretory responses, β -hexosaminidase in particular, without

Fig. 4. Effect of chlorpromazine on TPA-induced P47 phosphorylation and secretion

Gel-filtered platelets, pulse-labelled with [^{32}P]P₁, were incubated at 37 °C with 0.17 μM -TPA in the absence (●) and presence (○) of 30 μM -chlorpromazine. Samples were prepared for determination of P47 phosphorylation (a), β -hexosaminidase secretion (b) and ATP + ADP secretion (c). The phosphorylation of P47 was determined by phosphorylation method (b) (see the Materials and methods section) and is expressed as the ratio of the ^{32}P content in P47 to that in fraction A (Fig. 3).

Fig. 5. Effect of chlorpromazine on thrombin-induced P47 phosphorylation and secretion: dose-response relationship

The experiment was performed and the results were expressed as described in Fig. 4, except that the platelets were incubated for 30 s with the concentrations of thrombin shown: □, chlorpromazine present; ■, chlorpromazine absent.

affecting the progress of the accompanying P47 phosphorylation.

For reasons that are not clear, the protein phosphorylation caused by thrombin (Fig. 6a) was 12–16 times faster than that caused by TPA (Fig. 4a). We have

therefore compared the TPA-induced responses over a 16 min period, with the thrombin-induced responses occurring within 1 min, which gave similar shapes of the corresponding time-course curves for each agonist in the presence and absence of chlorpromazine (Figs. 4 and 6).

Fig. 6. Effect of chlorpromazine on TPA-induced P47 phosphorylation and secretion

The experiment was performed and the results were expressed as described in Fig. 4, except that 0.2 unit of thrombin/ml was used. Symbols are as in Fig. 5.

DISCUSSION

Using extracellular cytoplasmic adenine nucleotides to measure platelet lysis, we have demonstrated here that chlorpromazine causes lysis that is detectable at $50\text{ }\mu\text{M}$ and 70% at $500\text{ }\mu\text{M}$. Thrombin markedly enhanced this lysis to 20% at $50\text{ }\mu\text{M}$ - and 100% at $100\text{ }\mu\text{M}$ -chlorpromazine. Trifluoperazine, the piperazine analogue of chlorpromazine, behaved the same way (Holmsen *et al.*, 1984a). This is in contrast with the insignificant lysis below $200\text{ }\mu\text{M}$ -trifluoperazine reported by Tallant & Wallace (1985), who used extracellular lactate dehydrogenase to measure lysis. However, it has been established (Knight *et al.*, 1982; Authi *et al.*, 1986) that the platelet membrane may be perforated in such a specific way that nucleotides pass freely, whereas proteins such as enolase or lactate dehydrogenase do not. Thus, with adenine nucleotides as markers for lysis, a low- M_r permeability barrier is apparently distinguished from the high- M_r permeability barrier measured by enzyme proteins as markers. In the following, 'lysis' refers only to the low- M_r permeability barrier.

Chlorpromazine, trifluoperazine (Weiss & Levin, 1982) and W-7 (Nishikawa *et al.*, 1980) have been regarded as inhibitors of calmodulin. However, they also inhibited protein kinase C phosphorylation and ATP secretion, suggesting a central role of protein phosphorylations in the stimulus-secretion coupling (Feinstein & Hadjian, 1982; Kawahara *et al.*, 1980; Mori *et al.*, 1980). These studies were done with $100\text{ }\mu\text{M}$ or more of the inhibitors, which, according to our studies, cause severe lysis when thrombin is present. The inhibition of protein phosphorylation may therefore simply have been due to a general lytic effect of the phenothiazines, since we report here that non-lytic concentrations of chlorpromazine have no effect on the thrombin- or TPA-induced phosphorylation of P47, the substrate for protein kinase C.

Sanchez *et al.* (1983) demonstrated abolition of ATP secretion by a non-lytic ($30\text{ }\mu\text{M}$) concentration of chlorpromazine. We show here that non-lytic concentrations of chlorpromazine may inhibit both dense-granule secretion and acid hydrolase secretion without affecting phosphorylation of P47. This implies that P47 phosphorylation is not a necessary step in the stimulus-secretion coupling, or that chlorpromazine inhibits a step in the coupling process beyond P47 phosphorylation. Thrombin- and Ca^{2+} -induced platelet secretion that is independent of P47 phosphorylation has also been suggested from results with permeabilized platelets (Haslam & Davidson, 1984; Lapetina *et al.*, 1985).

Chlorpromazine alone caused a marked increase in $[\text{}^{32}\text{P}]\text{PIP}$, a moderate increase in $[\text{}^{32}\text{P}]\text{PIP}_2$ and a distinct decrease in $[\text{}^{32}\text{P}]\text{PI}$ without any formation of $[\text{}^{32}\text{P}]\text{PA}$. These results are similar to, although of lesser extent than, those reported by Tallant & Wallace (1985), using lytic doses of chlorpromazine. TPA alone also caused little change in $[\text{}^{32}\text{P}]\text{PI}$ and $[\text{}^{32}\text{P}]\text{PIP}_2$, but increased $[\text{}^{32}\text{P}]\text{PIP}$. These changes can be explained by activation of PI kinase and/or inhibition of PIP phosphomonoesterase, although, with TPA, with concomitant resynthesis of (the consumed) PI. Thrombin alone caused an initial, transient, decrease, followed by an increase in $[\text{}^{32}\text{P}]\text{PIP}_2$, a steady increase in $[\text{}^{32}\text{P}]\text{PIP}$ and a massive incorporation of ^{32}P into PI concomitant with accumulation of $[\text{}^{32}\text{P}]\text{PA}$. These changes have been interpreted as activation of the entire polyphosphoinositide cycle (Holmsen, 1985).

When chlorpromazine was used together with TPA or thrombin, which resulted in a marked inhibition of secretion, the increase in [32 P]PIP and/or [32 P]PIP₂ caused by the agonists alone was considerably enhanced. Thus inhibition of secretion by chlorpromazine is associated with increases in PIP and/or PIP₂. TPA was reported to inhibit thrombin-induced mobilization of Ca²⁺ (MacIntyre *et al.*, 1985) and diesteratic hydrolysis of PIP₂ (Rittenhouse & Sasson, 1985). The present studies, and those of de Chaffoy de Courcelles *et al.* (1984a), show that TPA alone markedly increases [32 P]PIP. It is therefore possible that the increase in PIP and PIP₂ causes inhibition of secretion.

The increase in [32 P]PIP + [32 P]PIP₂ caused by chlorpromazine in thrombin- or TPA-treated platelets was roughly the sum of the increase caused by each agent separately. This was strikingly similar to the additive effect of the drug on the thrombin- and TPA-induced phosphorylation of the two 80–90 kDa proteins. These phenomena may therefore be related, and suggest that the 80–90 kDa proteins represent one or more of the kinases and/or phosphatases which maintain the concentrations of PIP and PIP₂ and that the activity of these enzymes is regulated by phosphorylation. Chlorpromazine and other phenothiazines with anti-calmodulin effects are amphiphilic cations which interfere non-specifically with biological membranes (Bowley *et al.*, 1977; Sturton & Brindley, 1977; Zborowski & Brindley, 1983). These molecules are thought to intercalate among adjacent phospholipids in membrane monolayers, and, as the content of amphiphilic cation increases, a critical point is reached at which the membrane ruptures. Bowley *et al.* (1977) showed that the rate of the PA phosphatase reaction was decreased when phenothiazines were intercalated within the mixed micelles of PA (substrate) and phosphatidylcholine, and that this inhibition was proportional to the affinity of the various phenothiazines to the micelles; the inhibitory effect was thus thought to result from a decrease in the availability of substrate PA. It is therefore possible that the increase in [32 P]PIP (and [32 P]PIP₂) by chlorpromazine (concentrations below the rupture point) in our studies is due to an increase in the availability of PI (and PIP) in the membrane caused by this intercalation.

TPA is also an amphiphilic molecule, and its ability to increase [32 P]PIP may also be due to a non-specific intercalation. Indeed, thrombin causes a rapid formation of diacylglycerol (Rittenhouse-Simmons, 1979), which, like TPA, has been shown to increase PIP (and PIP₂) through interference with the kinase and phosphomonoesterase reactions (de Chaffoy de Courcelles *et al.*, 1984a,b). Part of the changes in the concentrations of PIP and PIP₂ caused by thrombin may therefore be due to a non-specific (intercalation) effect of the diacylglycerol produced.

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