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# Relationship between structure of phenothiazine analogues and their activity on platelet calcium fluxes

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**1** Phenothiazine analogues have been tested for their effect on calcium uptake into platelet membrane vesicles and on ionophore-induced platelet activation, both phenomena being $Ca^{2+}$-dependent.

**2** Both calcium uptake into membrane vesicles and ionophore-induced platelet activation were inhibited by the drugs.

**3** Evidence for two inhibitors as potent as chlorpromazine and trifluoperazine was found.

**4** These drugs are apparently competitive inhibitors of calcium uptake.

**5** A structure-activity relationship has been established.

**6** The data suggest that the phenothiazines are able to inhibit calmodulin-insensitive calcium uptake of platelet membrane vesicles and that therefore they cannot be assumed to be selective inhibitors of calmodulin interactions under all circumstances.

## Introduction

It is generally recognized that calcium ions play a key role in the regulation of platelet function (Detwiler *et al.*, 1978; Massini *et al.*, 1978). We have shown previously that in response to the calcium ionophore A 23187, ethylene diaminetetraacetic acid (EDTA)-treated platelets undergo an increase in light transmission. This is accompanied by a normal [$^{14}$C]-5-hydroxytryptamine release and thromboxane synthesis in the absence of aggregation: the stimulation of internal calcium fluxes by the ionophore A 23187 being sufficient to induce platelet activation (Lévy-Toledano *et al.*, 1982).

In the past few years, it has become clear that many of the regulatory effects initially attributed directly to $Ca^{2+}$ are in fact modulated by a ubiquitous protein: calmodulin (Klee *et al.*, 1980). Furthermore, this molecule was shown to be inactivated by phenothiazines such as trifluoperazine and chlorpromazine (Levin & Weiss, 1977; Weiss & Wallace, 1980). Some phenothiazines have been tested on platelet functions and found to inhibit platelet aggregation induced by various agonists (Kindness *et al.*, 1980; White & Raynor, 1980; Walenga *et al.*, 1981). Consequently, calmodulin has been suggested as a mediator of the action of $Ca^{2+}$ during platelet activation (Kambayashi *et al.*, 1981).

One of the main storage pools of calcium in the platelet is the dense tubular system (Massini *et al.*, 1978). This system is similar to the muscular sarcoplasmic reticulum which is able to incorporate and liberate calcium. A platelet membrane fraction which actively sequesters calcium has been isolated by Kaser-Glanzmann *et al.*, (1977) and chlorpromazine and calcium interactions have been demonstrated in rat synaptic membranes (Breton *et al.*, 1977). Chlorpromazine inhibits the calcium uptake of sarcoplasmic reticulum from smooth muscle of guinea-pig ileum (Chatuverdi *et al.*, 1978) and trifluoperazine inhibits the calcium transport into inside-out membrane vesicles of erythrocytes (Volpi *et al.*, 1981). We have studied the effect of 20 phenothiazine analogues on both platelet activation induced by the ionophore A 23187 and on the calcium uptake by platelet membrane vesicles, both phenomena being $Ca^{2+}$-dependent. The results led us to define some of the structural requirements for the inhibitory activity of the drugs and provide information on the way phenothiazines act in the platelet system.

## Methods

*Preparation of the vesicular membrane fraction*

A platelet membrane fraction which actively sequesters calcium was prepared by a modification of the method of Kaser-Glanzmann *et al.* (1977). These experiments were performed according to the principles of the declaration of Helsinki and informed consent was obtained. Blood was drawn from the antecubital vein of normal donors who had not ingested any drugs for two weeks and anticoagulated with 1/10 vol. (citric acid: 15.2 mM, trisodium citrate: 8.94 mM, monosodium phosphate $2H_2O$: 16.6 mM and glucose: 128 mM). Platelet-rich plasma (PRP) was separated by centrifugation at 120 *g* for 15 min at room temperature. Ethylene diaminetetraacetic acid (EDTA) was then added to the PRP to a final concentration of 5 mM and the platelets were pelleted by centrifugation at 3000 *g* for 15 min at room temperature. They were then washed twice with a modified Tyrode buffer containing (mM): NaCl 130, KCl 5, $NaH_2PO_4$ 1, $NaHCO_3$ 24, EDTA $Na_2$ 2, glucose 10, saccharose 12.5 and bovine serum albumin (BSA) 0.35% (w/v) and stored overnight at 4°C. The washed platelets were then centrifuged and resuspended in an homogenizing medium (mM): KCl 100, $MgSO_4$ 7 $H_2O$ 2, NaCl 25, citrate $Na_3$ 12, glucose 10, HEPES 25, adenosine triphosphate (ATP) 5 and bovine serum albumin (BSA) 0.35%, pH 7.5, 340 mosmol$^{-1}$. Following this step all the subsequent procedures were carried out at 4°C. Platelets were lysed by ultrasonication in a Branson sonifier $B_{12}$ at 40 watts. The ultrasonication was performed by 2 ml platelet fractions, ($2 \times 10^9$ platelets ml$^{-1}$), 4 times, for 5 s. The resulting platelet lysate was centrifuged at 19000 *g* during 25 min to eliminate granules, mitochondria and unlysed platelets then the supernatant was centrifuged at 100,000 *g* for 60 min. The pellet membrane fraction was resuspended in an incubation medium containing (mM): potassium oxalate 10, KCl 100, $MgCl_2$ 10, HEPES 20 and ATP 5, pH 7.55, stored at 4°C and used within 48 h to 72 h. Freezing at − 20°C involved a 50% loss of activity.

The protein concentration of the membrane fraction, 3 to 5 mg ml$^{-1}$, was determined by the Bradford method (1976) using gammaglobulins as standard. The recovery was around 300 μg for $10^9$ platelets.

*Calcium uptake measurement*

This was performed at 37°C using Millipore filters (pore size 0.45 μm, type HA) according to the method of Kaser-Glanzmann *et al.*, (1977) with slight modifications.

The platelet fraction to be tested (0.2 ml) was added to 1.8 ml of the incubation medium containing: KCl 100 mM, $MgCl_2$ 10 mM, potassium oxalate 10 mM as an anion precipitating agent, HEPES 20 mM, ATP 5 mM, pH 7.55 and various $^{45}CaCl_2$ concentrations up to 100 μM (1300–1500 cpm nmol$^{-1}$). Samples were stirred continuously at 37°C and 0.2 ml aliquots were withdrawn at intervals and filtered through membranes previously soaked in BSA 2 mg ml$^{-1}$. Filters were washed twice with 2 ml of 0.1 M $CaCl_2$ and counted in 10 ml of Unisolve scintillation liquid. Control experiments demonstrated that the filters retain no $^{45}Ca$ from the incubation medium itself. Calcium uptake was expressed as nmol $Ca^{2+}$ per mg protein. When calcium uptake measurements were performed in the presence of calmodulin (IBF, France), platelet membrane fraction was treated with EGTA in order to inactivate the calmodulin which is present in this preparation (Le Peuch *et al.*, 1983); 2 ml of platelet membranes was then incubated with 2 mM ethylene glycol bis (β-amino ethylether)-N, N′ tetracetic acid (EGTA) for 30 min at 37°C. The sample was diluted 10 fold in the incubation medium before centrifuging it at 100,000 *g* for 60 min. Platelet membrane vesicles were then resuspended in the same incubation medium.

*Effect of the inhibitors on the calcium uptake*

The inhibition pattern and the apparent inhibition constants were determined as follows: the reaction mixture containing different $^{45}CaCl_2$ concentrations was incubated for 30 min at 37°C in the presence or the absence of adequate doses of the inhibitors: the approx $IC_{50}$ (drug concentration producing 50% inhibition) was used, previously determined using 50 μM $CaCl_2$. Aliquots (0.2 ml) were withdrawn and treated as described previously. Calculations of apparent inhibition constants ($K_i$) were made using Lineweaver & Burk plots and represent the mean values of three to five independent experiments. All compounds tested were synthesized by Rhône-Poulenc (France) and were dissolved in water or in ethanol. This solvent has no measurable effect on calcium transport under the conditions used.

*Preparation of washed platelet suspensions using metrizamide gradients and measurement of ionophore-induced platelet activation by change in light transmission: (LT)*

Nine parts of blood collected by venepuncture was anticoagulated with one part of 0.077 M EDTA in saline. After centrifuging for 15 min at 120 *g* and room temperature, the platelet rich plasma (PRP) was transferred to a propylene tube with a plastic pipette and was stored at room temperature. The metrizamide gradient platelets (MGP) were pre-

**Table 1** Partial purification of accumulating calcium fraction from human platelets

| *Fraction* | *Total proteins (mg)* | *Total* activity (nmol min$^{-1}$)* | *Recovery (%)* | *Specific* activity (nmol min$^{-1}$ mg$^{-1}$)* | *Purification factor* |
|---|---|---|---|---|---|
| Lysate | 110 | 46.8 | 100 | 0.42 | 1 |
| 100,000 *g* pellet | 3.0 | 18.4 | 39 | 6.13 | 14.6 |

*Results are expressed as nmol calcium incorporated into membranes.

pared according to the method described by Lévy-Toledano *et al.*, (1979): 5 ml of PRP were layered onto a gradient composed of 1 ml of 25% (w/v) and 1 ml of 10% metrizamide and centrifuged for 15 min at 1000 *g* and 18°C. The platelet layer was resuspended in 4–5 ml of calcium-free buffer, pH 6, and deposited on a new metrizamide gradient at the same concentrations. After recentrifugation the platelet layer was resuspended in 4–5 ml of calcium-free buffer, pH 7.4: (NaCl 140 mM, $NaH_2PO_4$,$2H_2O$ 1.45 mM, $Na_2HPO_4$ 6.85 mM, glucose 11 mM in twice-distilled water). The calcium contamination was measured in the $Ca^{2+}$-free buffer using a Perkin Elmer 303 atomic absorption spectrophotometer and was found to be below 20 μM (detection limit of the apparatus).

The change in LT was measured in a Payton aggregometer at 37°C according to the method of Born & Cross (1963). To 0.5 ml of MGP (2 to 2.5 × $10^8$ platelets ml$^{-1}$) stirred at 1100 r.p.m., was added 3 μl of ionophore A 23187 (a gift from Lilly Laboratories, Indianapolis, USA) at a final concentration 0.3 μM in ethanol. This solvent has no measurable effect on platelets up to 1/100th of sample volume. The results are given in terms of the change in LT 3 min after the addition of ionophore, expressed as a percentage of the difference in LT between MGP and suspending buffer. In experiments involving the phenothiazine analogues, drugs were incubated with the MGP for 1 min at 37°C before the addition of ionophore. The $IC_{50}$ was determined for each drug.

## Results

### *Partial purification of the accumulating calcium membrane fraction*

Table 1 summarizes the recoveries, the specific activities and the purification factor of the subcellular fraction obtained during the isolation procedure. The calcium uptake activity of the 100,000 *g* pellet has a specific activity of 6,13 nmol mg$^{-1}$ min$^{-1}$ corresponding to a purification factor of 14.6 as compared to the lysate. The overall recovery of activity was 39%.

The calcium accumulating membrane fraction was characterized previously: the phosphodiesterase assay as well as the $^{125}$I-labelling using the lactoperoxidase-catalysed iodination revealed a contamination by plasma membranes. Further, the lysosomal and cytoplasmic contaminations were small as suggested by the β-glucuronidase and the lactate dehydrogenase assays (Le Peuch *et al.*, 1983). In addition, as revealed by the [$^{14}$C]-5 hydroxytryptamine ([$^{14}$C]-5-HT) labelling and the β-thromboglobulin assay, there was a small contamination by dense and α-granules (data not shown).

### *Characterization of the calcium uptake*

Figure 1 shows the time course of calcium uptake by vesicles as a function of different external calcium concentrations. The uptake was quite rapid and a steady state was reached within 60–90 min when the calcium concentration was within 50 to 100 μM.

By using the Lineweaver and Burk plot, (Figure 1, Inset) the results suggest that only one $Ca^{2+}$ transport system is involved in the $Ca^{2+}$ uptake into the vesicles. The apparent kinetics of calcium uptake were estimated: the initial rate of calcium uptake ($V_{max}$) was about 8 ± 4 nmol mg$^{-1}$ min$^{-1}$ and the highest accumulating capacity was 300 ± 100 nmol mg$^{-1}$ at 60 min in the presence of 100 μM $CaCl_2$. The apparent $K_m$ was about 45 ± 16 μM. The results of the apparent kinetics are the mean of 6 to 10 experiments.

The calcium uptake by membranes, which requires the presence of ATP, is greatly dependent on temperature (4°C < 20°C < 37°C), is enhanced by potassium oxalate and is also activated by 10 mM $MgCl_2$ (the Lineweaver–Burk plot shows a 3 fold increased affinity for the transport system, data not shown). Moreover the calcium uptake is mediated by a calcium-dependent, magnesium-activated adenosine triphosphatase ($(Ca^{2+} + Mg^{2+})$-ATPase) of 120,000 molecular weight (Le Peuch *et al.*, 1983).

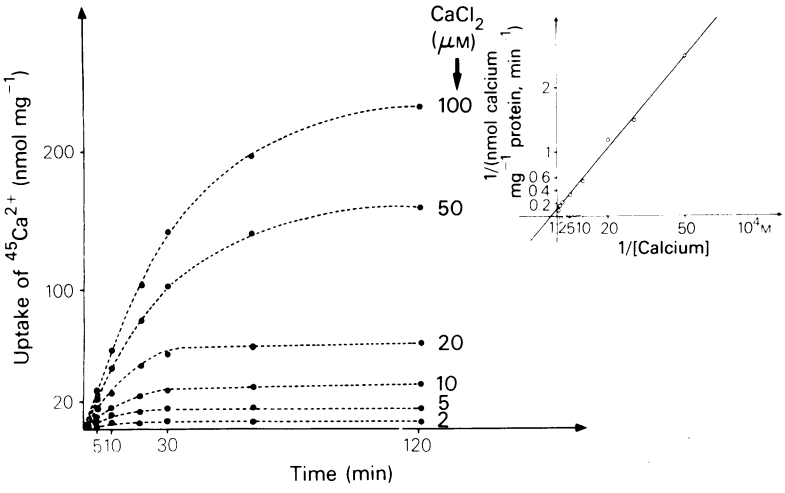



**Figure 1** Effect of different concentrations of $CaCl_2$ (2 to 100 μM) on calcium uptake by the 100,000 *g* fraction (180 μg $ml^{-1}$) enriched membrane vesicles. Inset: Lineweaver-Burk plot of another experiment. $^{45}Ca^{2+}$ uptake was measured as described in the Methods section.

All these data added to the fact that the accumulated $^{45}Ca^{2+}$ is released from the vesicles by the calcium ionophore A 23187 (Le Peuch *et al.*, 1983) show that $^{45}Ca^{2+}$ is indeed sequestered inside the vesicles and not bound to the membrane surface.

The possibility of calmodulin involvement in the regulation of $Ca^{2+}$ transport was examined by adding purified calmodulin to vesicles. Figure 2 shows that the amount of $^{45}Ca^{2+}$ taken up by the vesicles was never significantly modified at any tested $Ca^{2+}$ concentrations (10 to 100 μM). This could not reflect the presence of saturating amounts of calmodulin in the membranes as they were extensively washed with EGTA.

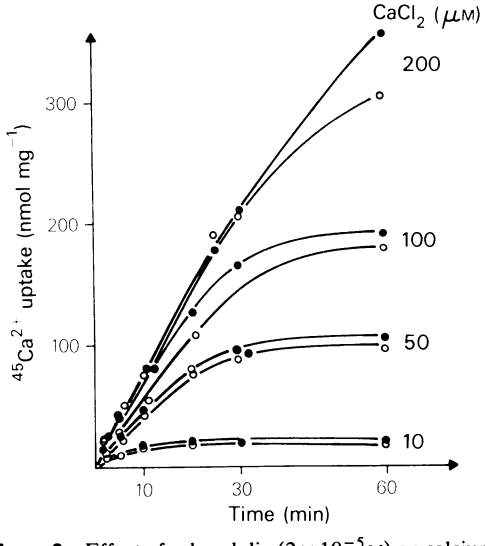



**Figure 2** Effect of calmodulin ($2 \times 10^{-5}$ M) on calcium uptake by the membrane vesicles at different $CaCl_2$ concentrations (10 to 200 μM). (●, ○): vesicles incubated at 37°C in the absence and presence of calmodulin, respectively.

*Effect of phenothiazine analogues on calcium movements*

We have studied the effect of more than 20 phenothiazine analogues modified on $R_1$, $R_2$ or $R_3$ (Table 2) on calcium uptake and ionophore-induced platelet activation.

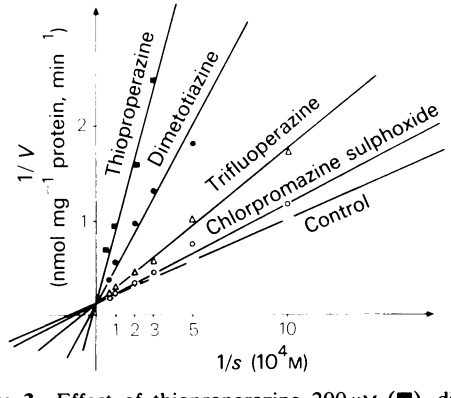
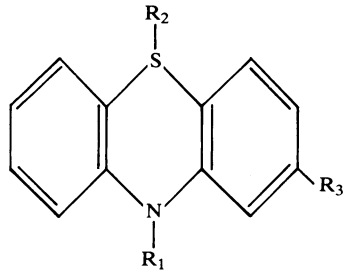



**Figure 3** Effect of thioproperazine 200 μM (■), dimetotiazine 200 μM (●), trifluoperazine 50 μM (△), and chlorpromazine sulphoxide 400 μM (○) on the calcium uptake of the vesicular membrane fraction using the Lineweaver-Burk plot.

**Table 2** Structure of phenothiazine analogues




| | No. | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| *Analogues modified on $R_1$* | | | | |
| Chlorpromazine (Cpz) | 1 | $(CH_2)N(CH_3)_2$ | | Cl |
| | 2 | $CH_2CH(CH_3)CH_2N(CH_3)_2$ | | Cl |
| | 3 | $CH_2C(CH_3)_2CH_2N(CH_2)_2$ | | Cl |
| | 4 | $CH_2CH(CH_3)CH_2N(C_2H_3)_2$ | | Cl |
| | 5 | $CH_2CH(CH_3)N(C_2H_3)_2$ | | Cl |
| Chlordiethazine | 6 | $(CH_2)_2N(C_2H_3)_2$ | | Cl |
| *Analogues modified on $R_1$ and $R_3$* | | | | |
| Promazine | 7 | $(CH_2)_3N(CH_3)_2$ | | H |
| Promethazine | 8 | $CH_2CH(CH_3)N(CH_3)_2$ | | H |
| Alimemazine | 9 | $CH_2CH(CH_3)CH_2N(CH_3)_2$ | | H |
| | 10 | $CH_2C(CH_3)_2CH_2N(CH_3)_2$ | | H |
| | 11 | $CH_2CH(CH_3)CH_2N(C_2H_5)_2$ | | H |
| Profenamine | 12 | $CH_2CH(CH_3)N(C_2H_5)_2$ | | H |
| Diethazine | 13 | $(CH_2)_2N(C_2H_5)_2$ | | H |
| Trifluoperazine | 14 | $(CH_2)_3N$⟨⟩$NCH_3$ | | $CF_3$ |
| Thioproperazine | 15 | $(CH_2)_3N$⟨⟩$NCH_3$ | | $SO_2N(CH_3)_2$ |
| Pipotiazine | 16 | $(CH_2)_3N$⟨⟩$CH_2CH_2OH$ | | $SO_2N(CH_3)_2$ |
| Dimetotiazine | 17 | $CH_2CH(CH_3)N(CH_3)_2$ | | $SO_2N(CH_3)_2$ |
| Propericiazine | 18 | $(CH_2)_3N$⟨⟩$OH$ | | CN |
| Metazionic acid | 19 | $CH_3$ | | $CH_2CO_2H$ |
| *Analogues modified on $R_1$ and $R_2$* | | | | |
| Sulphoxide (Cpz) | 20 | $(CH_2)_3N(CH_3)_2$ | O | Cl |
| Sulphone (Cpz) | 21 | $(CH_2)_3N(CH_3)_2$ | $O_2$ | Cl |
| *Analogues modified on $R_1$ and $R_2$ and $R_3$* | | | | |
| Oxomemazine | 22 | $CH_2CH(CH_3)CH_2N(CH_3)_2$ | $O_2$ | H |

*Calcium uptake* The inhibition pattern (Figure 3) and the inhibition constant (apparent $K_i$) (Table 3) were determined for each analogue.

The Lineweaver-Burk plot allows us to establish that all the active molecule tested appear as apparent competitive inhibitors in respect to calcium. As an example, Figure 3 shows the inhibition induced by trifluoperazine, thioproperazine, dimetotiazine and chlorpromazine sulphoxide. The first three drugs appear to be much more inhibitory than the inactive metabolite of chlorpromazine (sulphoxide).

The inhibition induced by the drugs is reversed by $CaCl_2$ but not by calmodulin. Figure 4a shows that $CaCl_2$ added before the steady state can overcome the inhibition of the $Ca^{2+}$ uptake induced by either chlorpromazine, trifluoperazine or compounds 2 and 4. The rate of $Ca^{2+}$ reuptake in the presence of the drugs or in their absence (control) appears to be nearly equal. In contrast, after addition of an excess of calmodulin ($2 \times 10^{-5}$ M) before the steady state no reversibility is observed (Figure 4b).

**Table 3** Inhibition of calcium uptake and platelet activation by phenothiazine analogues

| no. | Analogue | Activation $IC_{50}$ (μM) | Calcium uptake $K_i$ (μM) |
|---|---|---|---|
| 2 | | 19±2* | 62±13* |
| 14 | Trifluoperazine | 18±2 | 71±9 |
| 1 | Chlorpromazine (Cpz) | 33±6 | 75±39 |
| 4 | | 23±10 | 85±15 |
| 15 | Thioproperazine | 51±6 | 100±39 |
| 8 | Promethazine | 100±20 | 104±10 |
| 17 | Dimetotiazine | 99±7 | 128±24 |
| 6 | Chlordiethazine | 81±22 | 130±35 |
| 18 | Propericiazine | 112±26 | 135±30 |
| 3 | | ND | 149±21 |
| 11 | | ND | 159±72 |
| 16 | Pipotiazine | 72±19 | 176±61 |
| 5 | | 56±5 | 229±56 |
| 13 | Diethazine | 50±3 | 241±112 |
| 10 | | ND | 274±139 |
| 12 | Profenamine | 60±12 | 353±33 |
| 7 | Promazine | 72±12 | 501±229 |
| 9 | Alimemazine | 41±6 | 690±247 |
| 21 | Sulphone (Cpz) | ∞ | 865±405 |
| 22 | Oxomemazine | ∞ | 2032±1147 |
| 20 | Sulphoxide (Cpz) | ∞ | 2051±158 |
| 19 | Metazianic acid | ∞ | 2168±1200 |

Results are the mean of 3 to 5 experiments.
*Mean ± s.e.mean. ND = not done.

*Ionophore-induced platelet activation* MGP underwent an increase in light transmission (Figure 5, inset) in the presence of ionophore A 23187 0.3 μM. We have determined the $IC_{50}$ of the different drugs on the light transmission change as shown in Figure 5 (for 4 of them) and in Table 3 (for all of them).

Figure 5 shows that the analogue no. 4 and chlorpromazine are potent inhibitors ($IC_{50}$ = 23 and 33 μM respectively) while dimetotiazine is a moderate inhibitor ($IC_{50}$ = 99 μM) and chlorpromazine sulphoxide is ineffective.

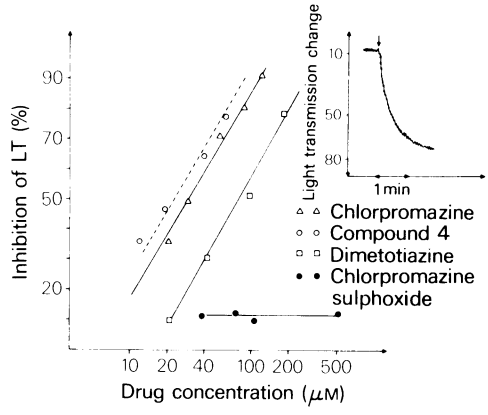



**Figure 5** Effect of some analogues on the ionophore (0.3 μM)-induced light transmission (LT) change of washed platelet on metrizamide gradients (MGP). The $IC_{50}$ was estimated by varying the concentration of the drugs (from 10 to 500 μM) and calculating the concentrations required for 50% inhibition of the LT intensity obtained in the absence of the drug 2 min after the addition of A 23187. (△) Chlorpromazine; (○) compound 4; (□) dimetotiazine; (●) chlorpromazine sulphoxide. Inset: Ionophore-induced LT change of the MGP in function of the time.

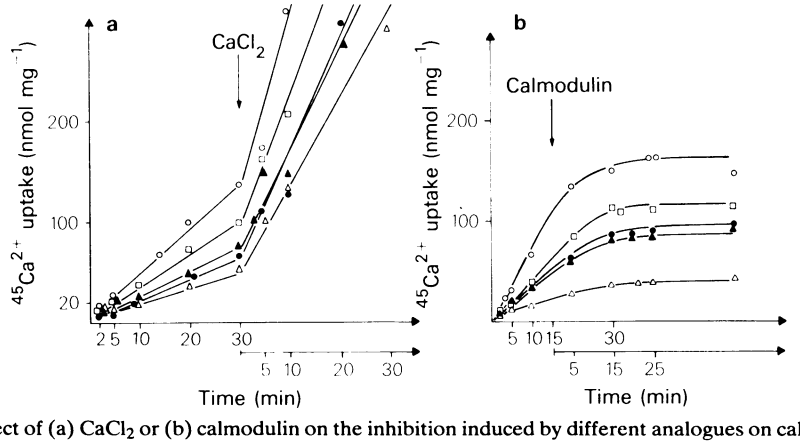



**Figure 4** Effect of (a) $CaCl_2$ or (b) calmodulin on the inhibition induced by different analogues on calcium uptake by membrane vesicles. Technical details are in the Methods section. Vesicles were incubated in the absence (○) or in the presence of chlorpromazine 75 μM (□), compound 4 100 μM (▲), trifluoperazine 25 μM (△) or compound 2 100 μM (●). At the indicated times $CaCl_2$ (200 μM) (a) or calmodulin ($2 \times 10^{-5}$ M) (b) was added to the vesicles.

*Relative inhibitory potency of the 22 phenothiazine analogues* is shown in Table 3 where the drugs are classified according to their $K_i$ values. This classification can also be taken into account for the inhibitory activity on the ionophore-induced platelet activation. Indeed when we compare the drug-induced inhibitory activity in both systems it appears that the four most potent inhibitors on platelet activation (compounds no. 2, 14, 1, 4) are also potent inhibitors of calcium uptake. Further, the drugs which do not modify platelet activation (compounds no. 21, 22, 20, 19) are weak or inactive inhibitors of calcium uptake into membrane vesicles. The other compounds can be grouped into moderate inhibitors for both systems.

*Structure-activity relationship*

With reference to Table 2 we observe that the best inhibitors (compounds 2, 14, 1, 4) have a substitution in $R_3$ with high steric hindrance (Cl or $CF_3$). Furthermore these compounds have a linear chain of three carbons (compound 1, 14) or a chain of four carbon atoms with one methyl group substituted (compounds 2 and 4) on $R_1$. The replacement of methyl groups by ethyl on nitrogen does not alter the activity (compounds 2 and 4).

All these compounds have an affinity of the same magnitude for calcium transport and they all inhibit ionophore-induced platelet activation with an $IC_{50}$ of 30 $\mu$M. Several chemical modifications are found in the group of moderate inhibitors: compounds with the Cl atom substituted by various groups (compounds 15, 8, 10, 12, 13, 17 and 18); and those where the carbon chain can be substituted either by two atoms (compound 6) or three atoms in a linear chain (compounds 15, 16, 18) or ramified chain (compounds 5, 8, 17) or four to five atoms in a ramified chain (compounds 11 and 3).

Finally the group of weak or inactive inhibitors (compounds 21, 22, 20, 19) contains an analogue with a very short carbon chain in $R_1$ (compound 19), and three analogues with an oxidation on the sulphur atom in $R_2$ (compounds 21, 22, 20).

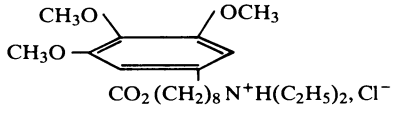
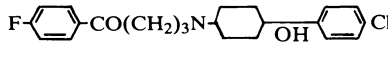
In conclusion, active drugs have a Cl atom or other high steric hindrance substitutions on $R_3$. The importance of this substitution is confirmed when we compare the activity of, for example, compounds 1 and 7, 2 and 8, 3 and 9, 4 and 10, 6 and 12 in which the last of each pair is modified by the Cl atom substitution. From the carbon chain substituted in $R_1$, it appears that the best inhibitory activity is obtained by structures made of three to four atoms. Furthermore, the oxidation of the sulphur atom decreased the inhibitory effect. Concerning the inhibition of the ionophore-induced platelet activation, the same conclusions can be drawn for the structure-activity relationship for these analogues.

Among the drugs tested on either calcium uptake by membrane vesicles or on ionophore-induced platelet activation, 4 phenothiazine analogues are found to be potent inhibitors: trifluoperazine (compound 14), compounds 2 and 4, and chlorpromazine (compound 1).

*Effect of non phenothiazine compounds known to interfere with either internal calcium or calmodulin*

Table 4 shows that 8-(*N*, *N*-diethylamino)-octyl, 3, 4, 5 trimethoxybenzoate ($TMB_8$), a calcium antagonist, (Chiou & Malagodi, 1975) is much less effective than the phenothiazine compounds 2, 14, 1 and 4, on either calcium uptake or on ionophore-induced platelet activation ($IC_{50} = 200$ to 500 $\mu$M). Haloperidol, a butyrophenone with calmodulin affinity (Weiss & Wallace, 1980) is also ineffective ($IC_{50} = 1$ mM).

**Table 4** Effect of non phenothiazine compounds on ionophore-induced platelet activation and calcium uptake

| *Compound* | *Structure* | *Activation* (mM)* | *Calcium uptake* (mM)** |
|---|---|---|---|
| $TMB_8$ |  | 0.2 | 0.5 |
| Haloperidol |  | > 0.4 | > 1 |

*Results are expressed as $IC_{50}$

**$IC_{50}$ was obtained using 50 $\mu$M calcium.

## Discussion

Phenothiazines inhibit platelet aggregation and secretion induced by various agonists (Kindness *et al.*, 1980; White & Raynor, 1980; Walenga *et al.*, 1981). We describe here the effects of more than 20 phenothiazine analogues on ionophore A 23187-induced platelet activation and calcium uptake by platelet membrane vesicles.

Phenothiazines previously described as inhibitors of platelet aggregation and secretion were also found to be inhibitors of the ionophore-induced platelet activation in the absence of aggregation and of the calcium uptake by platelet membranes.

Two phenothiazine analogues (4 and 2) were as potent inhibitors on both platelet activation and calcium uptake as are chlorpromazine and trifluoperazine. The effects of 22 phenothiazine analogues established a structure-activity relationship. Structural requirements were: no oxidation of the sulphur atom on $R_2$; a Cl substituent or another high hindrance group on $R_3$; and a relatively short side chain on $R_1$. Non-phenothiazine drugs such as $TMB_8$ which inhibits internal calcium release (Chiou & Malagodi, 1975) were much less potent than phenothiazines. Further, we observed an apparent correlation between the phenothiazine-induced inhibition of platelet activation and calcium uptake, both phenomena being calcium-dependent (Detwiler *et al.*, 1978; Massini *et al.*, 1978; Lévy-Toledano *et al.*, 1982).

This correlation is based partly on the fact that some of these analogues (compounds 4, 2, chlorpromazine and trifluoperazine) are as good inhibitors for ionophore-induced platelet activation as for calcium uptake. Besides, the compounds 21, 22, 20 and 19 which do not modify platelet activation are also inactive on calcium uptake. The other compounds can be considered as intermediate inhibitors for both systems.

Finally, our results establish that the inhibition of calcium uptake by phenothiazine analogues is apparently competitive. This suggests a complex mechanism of action for phenothiazines.

Many calcium effects appear to be exerted by the complex calcium-calmodulin (Cheung, 1980) including the $(Ca^{2+} + Mg^{2+})$-ATPase of the plasma membrane. Levin & Weiss (1977) found that phenothiazines inactivate this complex by binding to calmodulin in a calcium-dependent fashion. The question is whether inhibition is mediated only through competition with calcium or by inactivation of calmodulin. Several findings are in agreement with calmodulin being involved in our system: (i) Calmodulin is present in the membrane preparation: 0.5% (w/w) of the proteins in the 100,000 *g* fraction (Le Peuch *et al.*, 1983); (ii) Some phenothiazine analogues have been tested for their ability to displace [$^3$H]-trifluoperazine from calmodulin (Weiss & Wallace, 1980) and the most active drugs were found to be trifluoperazine, chlorpromazine, promethazine and chlorpromazine sulphoxide, the latter being significantly less potent; these results are in agreement with the relative reactivity of the phenothiazine analogues on either $Ca^{2+}$ uptake or on ionophore-induced platelet activation; (iii) Concerning the ionophore-induced platelet activation, it has been shown that the change in light transmission was accompanied by thromboxane synthesis and a release of [$^{14}$C]-5-HT (Lévy-Toledano *et al.*, 1982). Thromboxane synthesis involves phospholipase stimulation and a recent work suggests that phospholipase $A_2$ may require calmodulin as activator (Walenga *et al.*, 1981). Calmodulin may also be involved in the ionophore-induced platelet secretion: Adelstein & Conti (1972) have shown that the phosphorylation of light chain myosin increases the activity of actin-activated myosin ATPase and presumably the resultant actomyosin contraction mediates the release reaction. Recently the platelet light chain myosin kinase has been identified as a $Ca^{2+}$-dependent enzyme requiring calmodulin for its activity (Hathaway & Adelstein, 1979; Nishikawa *et al.*, 1980).

However, some results do not agree with the involvement of calmodulin in calcium uptake. (i) Calmodulin does not interfere directly with the $(Ca^{2+} + Mg^{2+})$-ATPase of internal membranes: Le Peuch *et al.*, (1979) have shown that calmodulin increases calcium uptake by the cardiac sarcoplasmic reticulum but has no effect on $(Ca^{2+} + Mg^{2+})$-ATPase. Thus calmodulin would not be a complete subunit of the calcium pump, as suggested for red cell membranes (Vincenzi *et al.*, 1980), but would react as a regulator by the activation of the $Ca^{2+}$-calmodulin-dependent protein kinase which phosphorylates the phospholamban, thereby increasing calcium uptake.

In the membrane vesicles used in this study the addition of calmodulin does not stimulate the calcium-uptake: this is in agreement with the absence of calmodulin binding to the $M_r$ 120,000 enzyme as revealed by the gel overlay technique (Le Peuch *et al.*, 1983). This result is further strengthened by the lack of significant stimulation by calmodulin of the platelet membrane $Ca^{2+}$-ATPase (Dean & Sullivan, 1982). Further a 23 K dalton protein (different from phospholamban) is phosphorylated in the presence of the catalytic subunit of the cyclic AMP-dependent protein kinase; but we have been unable to demonstrate phosphorylation mediated by a $Ca^{2+}$-calmodulin dependent protein kinase (Le Peuch *et al.*, 1983). (ii) Inhibition of calcium uptake by phenothiazines could not be reversed by an excess of calmodulin but was reversed by calcium. (iii)

Haloperidol which binds to calmodulin (Weiss & Wallace, 1980) had no effect on either calcium uptake or platelet activation by ionophore. This has also been observed for the calmodulin activation of $(Ca^{2+} + Mg^{2+})$-ATPase of red blood cell membranes (Vincenzi & Hinds, 1980).

On the basis of these results, and since we cannot exclude the role of calmodulin in the phenothiazine-induced inhibition of platelet activation, the apparent competitive antagonism between calcium and phenothiazines, during calcium uptake, means that these drugs cannot be assumed to exert their effect by interacting with calmodulin at least in this system.

A growing number of results suggest now that phenothiazines are apparently not specific for calmodulin (Wreen *et al.*, 1981; Corps *et al.*, 1982; Ruben & Rasmussen, 1982; Luthra, 1982). This is emphasized by the studies on the purified reconstituted ATPase of red blood cells, where trifluoperazine prevented not only the activation of the reconstituted ATPase by calmodulin but also that produced by phosphatidylserine and by controlled proteolysis (Carafoli & Zurini, 1982). The role of phenothiazines on the platelet $(Ca^{2+} + Mg^{2+})$-ATPase is now under investigation.

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