Effects of psychotropic drugs on the thrombin-induced liberation of arachidonate in human platelets

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ABSTRACT

الأهداف: لمقارنة آثار عقار كلوربرومازين (CPZ)، عقار بروكلوربيرازين (PCP)، عقار تريفلوبيرازين (TFP)، عقار كلوزابين (CLO)، عقار هالوبيريدول (HPD)، عقار كويتيابين (QTP)، عقار بيموزيد (PMZ)، وعقار أولانزابين (OLP)، بالإضافة لمضادات الإكتئاب أميتريبتيلين (AMI)، آيمبرامين (IMI)، نوتريبتيلين (NTP) على تحرير حمض أراكيندونيت المحرض بالثرومبين في الصفيحات البشرية (AA).

الطريقة: أجريت هذه الدراسة بقسم الطب الإحيائي – جامعة بيرجون – النرويج، خلال الفترة مابين عام 2006م وحتى 2007م. تم حضن الصفيحات البشرية المصنفة بالحمض أراكيندونيت (^3H) بالثرومبين في غياب ووجود العقاقير، وتم تحديد كمية خالية من حمض أراكيندونيت (^3H) المحرر. تم تحديد فسفرة سلسلة الميوسين الخفيفة (MLC) في (^{32}P) والصفيحات المصنفة بالفوسفيت بعد (PACE) – (SDS). تم تحديد آثار العقاقير على المنطقة الجزيئية وضغط السطح لطبقات الدهون الفسفورية في جهاز لانجمور.

النتائج: خفضت جميع العقاقير تحرير حمض أراكيندونيت حسب الترتيب التالي:
OLP<QTP<HPD<AMI<IMI<PMZ<NTP<CPZ<TFP<PCP. نظراً لكون عقار فينوثيازين مضاد للكالمودولين، فهذه الخاصية للعقاقير تم اختبارها كإرجاع محرض بالثرومبين لفسفرة سلسلة الميوسين الخفيفة (MLC) في ^{32}P Pi المصنف للصفيحات . فقط (TFP)، (CPZ)، (PCP) و(NTP) خفضوا من (MLC) فسفرة سلسلة الميوسين الخفيفة. جميع العقاقير الإحدى عشر التي تمت دراستها زادت بشكل ملحوظ من قيمة المنطقة الجزيئية لطبقات الدهون الفسفورية بنسبة 37 درجة.

خاتمة: يعتقد أن ميكانيكية تقليص تحرير حمض أراكيندونيت تتداخل مع تفاعل الفوسفات الخلوي ($cPLA_2$) (A_2) للتركيبة الفوسفرورية للدهون (PLA_2) في أغشية الصفيحات.

Objective: To compare the effects of chlorpromazine (CPZ), prochlorperazine (PCP), trifluoperazine (TFP), clozapine (CLO), haloperidol (HPD), quetiapine (QTP), pimozide (PMZ), and olanzapine (OLP) as well as the tricyclic antidepressants amitriptyline (AMI), imipramine (IMI), and nortriptyline (NTP) on thrombin-induced liberation of arachidonic acid (AA) in platelets.

Methods. This work was carried out at the Department of Biomedicine, University of Bergen, Norway in 2006-2007. Human platelets pre labelled with [^{3}H] arachidonate were incubated with thrombin in the absence and presence of the drugs, and the amount of free [^{3}H] arachidonate liberated was determined. Myosin light chain (MLC) phosphorylation was determined in [^{32}P] phosphate-labelled platelets after sodium dodecyl sulfate polyacrylamide gel electrophoresis. The effects of the drugs on the molecular area and surface pressure of phospholipid monolayers were determined in the Langmuir apparatus.

Results. All drugs reduced arachidonate liberation with the ranking order of increasing potency: OLP <QTP<HPD<AMI<IMI<PMZ<NTP<CPZ<TFP<PCP. Since phenothiazines are calmodulin antagonists, this property of the drugs was tested as reduction of thrombin-induced phosphorylation of MLC in [^{32}P] Pi-labelled platelets. Only PCP, CPZ, TFP, and NTP reduced MLC phosphorylation. All 11 drugs studied markedly increased the mean molecular area of dipalmitoyl phosphatidylserine monolayers at 37^{0}C.

Conclusions. The mechanism(s) for reduction of arachidonate liberation is thought to interfere with activation of cytosolic phospholipase A_2 ($cPLA_2$) by alteration of the PLA_2 phospholipid substrate structure in platelet membranes.

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Psychoactive drugs that selectively modify central nervous system (CNS) functions often affect several of these to varying degrees.[1] All antipsychotic or neuroleptic agents, which include tricyclic phenothiazines, thioxanthenes, dibenzepines, butyrophenones, and other heterocyclics are considered to achieve their pharmacological effects by blocking D2 dopaminergic receptors, although some also interact with D1 dopaminergic, serotonergic, and alpha-adrenergic receptors.[2] Lipid signalling pathways in blood platelets, which lack D2 receptors,[3] are affected by antipsychotic drugs. Phospholipase C is both stimulated and inhibited in a hormetic[4] manner by trifluoperazine (TFP) and several other antipsychotic agents.[5] Prochlorperazine (PCP), TFP, haloperidol (HPD), clozapine (CLO), pimozide (PMZ), and quetiapine (QTP) also interfere with polyphosphoinositide metabolism.[6] Physicochemical studies showed that chlorpromazine (CPZ),[7] and TFP[8] increase the molecular area in monolayers of negatively charged glycerophospholipids such as phosphatidylserine. Furthermore, CPZ causes marked changes in the structure of liposomes containing phosphatidylcholine (PS),[9] suggesting that CPZ intercalates in the membranes. A similar interaction of other amphiphilic, cationic drugs with negatively charged substrate polyphosphoinositides, would explain its effects on polyphosphoinositide metabolism.[6] Another lipid signalling pathway involving cytosolic phospholipase A_2 (cPLA$_2$) is inhibited in platelets by TFP,[10] and other amphiphilic drugs.[11-15] The PLA$_2$ inhibition appears not to be associated with the interaction of the drugs with phosphatidylserine since thrombin activation of platelet PLA$_2$ involves mostly PS as substrate.[10] This has shown little interaction with the drugs when tested with monolayers[7] and liposomes.[16] The PLA$_2$ from rat brain cortex[17] and recombinant PLA$_2$[18] have also been inhibited by cationic amphiphiles in assay systems with substrates present in membranes. Cytosolic PLA$_2$ is generally believed to be regulated by several processes including translocation from cytosol to the plasma membrane in a Ca^{2+}-dependent manner and then phosphorylated by mitogen-activated, Ca^{2+}/calmodulin-dependent, and/or other protein kinases.[19] Since the phenothiazines are well known to act as calmodulin antagonists,[20] the other psychotropic drugs may also act in a similar fashion, although it has been claimed that PLA$_2$ in rat platelets is dependent on Ca^{2+} mobilization in a calmodulin-independent manner.[21] In the present paper, we have investigated whether the cationic amphiphilic psychotropic drugs CLO, olanzapine (OLP), HPD, PMZ, QTP, CPZ, TFP, PCP, nortriptyline (NTP), amitriptyline (AMI) and imipramine (IMI), also cause inhibition of PLA$_2$ activity in thrombin-activated human platelets, measured as liberation of free arachidonate. To distinguish whether these agents act via a calmodulin-dependent mechanism, we also measured the effect of the drugs on myosin light chain (MLC) phosphorylation, which in human platelets is totally dependent on calmodulin[22] and activated by thrombin[23] in parallel with PLA$_2$.[10]

Methods. *Chemicals.* All drugs used in this work were pure chemicals. The CPZ, TFP, NTP, and IMI, were obtained from Sigma (St. Louis, Missouri, USA). The HPD and PMZ were from Janssen Foundation (Berse, Belgium), AMT from Lundbeck (Copenhagen, Denmark), QTP was from Astra Zeneca (UK Ltd. Macclesfield, UK), PCP was from Rhone-Poulenc Pharma Norden (Oslo, Norway), CLO was from Alpharma AS (Oslo, Norway), and OLP from Eli Lilly & Co (Indianapolis, USA). The chemical structures of the drugs are shown in **Figure 1**. Dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylserine (DPPS) were obtained from Avanti Polar Lipids (Birmingham, AL). The [5,6,8,9,11,12,14,15-^{3}H] Arachidonic acid (code TRK757, 212 mCi/ml) in ethanol was obtained from Amersham-Pharmacia Biotech (Buckinghamshire, UK). Thrombin (bovine) was from Parke-Davis (Scarborough, Ontario, Canada) and stored in small portions of 100 U/ml in 0.15 M sodium chloride (NaCl) at -20°C; appropriate dilutions with 0.15 M NaCl were made before each experiment and the rest of the stock portion was discarded. The CPZ, TFP, PCP, QTP, AMT, NTP, and IMI were dissolved in 0.15 M NaCl, the stock solutions were stored in the dark at -20°C and dilutions were made with 0.15 M NaCl immediately prior to use. The CLO, OLP, HPD, and PMZ were dissolved

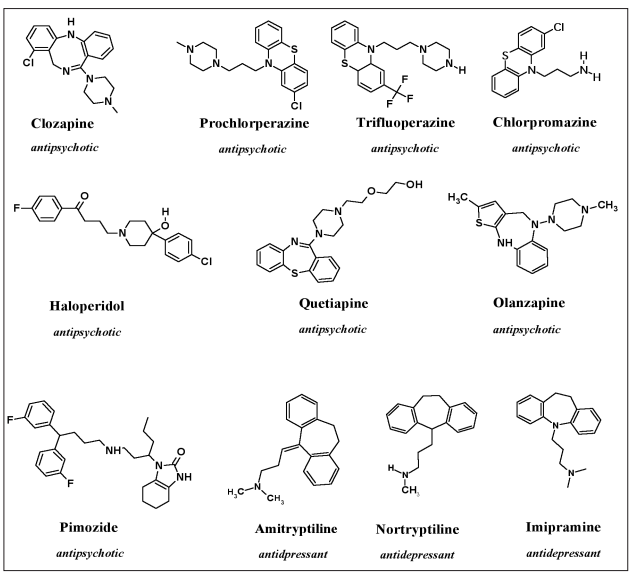

Figure 1 - Chemical structures of the drugs used.

in dimethylsulfoxide (DMSO) and the stock solutions were stored in the dark at -20°C and dilutions made with 0.15 M NaCl for CLO, OLP, HPD, or a Tyrode's solution containing 0.2% albumin for PMZ just before experiments were to be performed.

Blood. Blood was collected at the blood bank, Haukeland University Hospital, Bergen, Norway, and anticoagulated with acid citrate dextrose ("ACD", 85 mM Na_3 citrate, 71 mM citric acid, 111 mM dextrose; one volume + 4 volumes of blood) from regular donors and from therapeutic phlebotomy hemochromatosis patients who claimed not to have taken any medications 10 days prior to blood donation. The blood collection was carried out according to Norwegian rules and regulations in force and according to an earlier permit from the Ethics committee and with informed consent from the blood donors.

Preparation of [^{3}H] AA-labelled, gel-filtered platelets (GFP). Concentrated platelet-rich plasma (PRP) was prepared from the blood as described elsewhere,[5,6] and incubated with 2.5µ Ci/ml PRP [^{3}H] AA at 37°C for 2 hours and then transferred by gel-filtration into Tyrode's solution, containing 0.4% bovine albumin and 5 mM glucose. The process involves passing the PRP through a column of Sepharose 2B with Tyrode's solution at room temperature; the platelets are excluded from the gel particles and elute free from plasma immediately after the void volume. The platelet count in radioactive GFP was adjusted to 3.5 x 10^8 cells/ml with the Tyrode's solution, and the cells were used immediately in the experiments.

Preparation of [^{32}P] Pi-labelled platelets. Concentrated PRP was incubated with 0.2 mCi [^{32}P] Pi/ml at 37^{0}C for one hour and then transferred by gel-filtration into Tyrode's solution without phosphate and calcium and containing 0.1% of bovine albumin and 5 mM glucose. The radioactive GFP was adjusted to 3.5 x 10^8 platelets/ml.

Thrombin-induced activation of PLA_2. Portions (5 ml) of GFP containing [^{3}H] AA-labelled platelets were pre-incubated at 37^{0}C for 15 minutes. They were then incubated in the presence of drugs (at various concentrations) or their solvents (500 µl of each) for 120 seconds before thrombin (500 µl, final concentrations are specified for each type of experiment in the figure legends) was added and the incubation was continued. At the times indicated (after thrombin addition) aliquots (500 µl) of the incubation mixtures were added to 2 ml ice-cold chloroform/methanol. Ice-cold chloroform and water (500 µl of each) was then added, the phases allowed to separate, and the chloroform phases were evaporated to dryness under a N_2 jet as described elsewhere.[24] The dried lipids were dissolved in 40 µl chloroform, applied to silica thin-layer plates (Silicagel 60, Aluminium, Merck, Darmstadt, Germany) and chromatographed for 90 minutes at room temperature with the upper phase of ethyl acetate/iso-octane/glacial acetic acid/ ddH_2O (90:50:20:100). In this chromatography system, the phospholipids remain at the application point while free AA had an R_f of 0.73. Small amounts of prostaglandins and thromboxanes amounting to less than 0.6% of the total [^{3}H] AA incorporated in the platelet phospholipids, were produced, but this was ignored. The radioactive spots were detected and quantified with a Raytest Radio-TLC Analyzer (RITA-90, Raytek Scientific, Sheffield, UK).

Thrombin-induced phosphorylation of 20-kDa myosin light chain (MLC). Portions of 500 µl [^{32}P] Pi-labelled GFP were pre-incubated at 37^{0}C following addition (50 µl) of the drugs (30 µM, final concentration) or their solvents (50 µl) for 90 seconds. Thrombin (0.45 U/ml, final concentration) was added and the incubation continued for a further 6 minutes. To stop the reaction, 150 µl of the incubation mixture was mixed with 75 µl 4xLaemmli denaturing buffer.[25] The mixture was vortexed, sonicated for 2 minutes, centrifuged at 12.000 rpm for 3 minutes and the supernatants heated for 5 minutes at 95°C. The samples were subjected to SDS-PAGE (12% separating gel; 4% stacking gel) at 68 V, 500 mA (250 watt) for 18 hours at room temperature, and the radioactive bands were visualized and quantified (as pixels) in a Phosphoimager (Biorad).

Langmuir monolayer experiments. The experiments were carried out with a KSV Minitrough (Helsinki, Finland) with MilliQ water as the subphase. The calculation of percent change in mean molecular area at surface pressures of 10, 20m and 30 mN/m for the drug-containing sample relative to the corresponding area in the control without drug at the same surface pressures was performed as described by Broniec et al.[8]

Statistical analysis. The PLA_2 mean value per donor and drug was obtained by averaging the duplicates for all 3 donors. Then, a 2-tailed t-test comparing the means was conducted by comparing each of the values for CLO, OLP, HPD, and PMZ to the control with DMSO. The same approach was applied to QTP, CPZ, TFP, PCP, NTP, AMI, and IMI by comparing them to the control with NaCl. The obtained p-values thus indicate if there were a statistical change upon administration of the drug, compared to the control. For MLC, the respective control value (MLC radioactivity in platelets not treated with thrombin) for each donor was subtracted from the obtained values (with thrombin) and the results for CLO, OLP, HPD, and PMZ were compared to the DMSO control by a 2-tailed t-test comparing the means. The same approach was applied to QTP, CPZ, TFP, PCP, NTP, AMI, and IMI, and the results were

thereafter compared to the NaCl control using the same 2-tailed t-test. The obtained p-values indicate whether there was a significant difference between the drug treated samples and the respective controls. Each drug was tested with platelets from 3 different donors.

Results. Effects of thrombin concentration, incubation time, and concentration of drugs on the liberation of [^{3}H]AA. When [^{3}H]AA-labelled GFP was incubated with increasing concentrations of thrombin for 12 minutes in the absence of drugs, the amount of liberated free [^{3}H]AA increased sharply up to 0.12 U/ml of thrombin, and further increase in the thrombin concentration produced only slightly more [^{3}H]AA (results not shown). Most of the experiments described below were carried out with 0.25 U/ml of thrombin. Incubation of [^{3}H]AA-labelled platelets with thrombin for increasing times in the absence of drugs resulted in a rapid production of free [^{3}H]AA, which culminated after 6-8 minutes and decreased slowly thereafter (**Figure 2**). The proportion of free [^{3}H]AA produced maximally by 0.25 U/ml of thrombin was 9-14% of the total [^{3}H]AA incorporated in the platelet phospholipids (data not shown, platelets from 11 donors). However, the maximal free [^{3}H]AA produced by 0.25 U/ml of thrombin varied among platelets from different donors. As demonstrated by the control curves in **Figure 2**, the maximal [^{3}H]AA liberated varied between 2500-7000% of the amount of the free [^{3}H]AA in control platelets not treated with thrombin. The effects of the 11 psychotropic drugs on the time course of thrombin-induced [^{3}H]AA liberation were tested with drug concentrations of 10, 20, and 30 μM, and with platelets from different donors for each drug. **Figure 2** shows the effects of PCP, PMZ, QTP, and CLO, where each drug was tested with platelets from different donors. It can be seen that each drug reduced

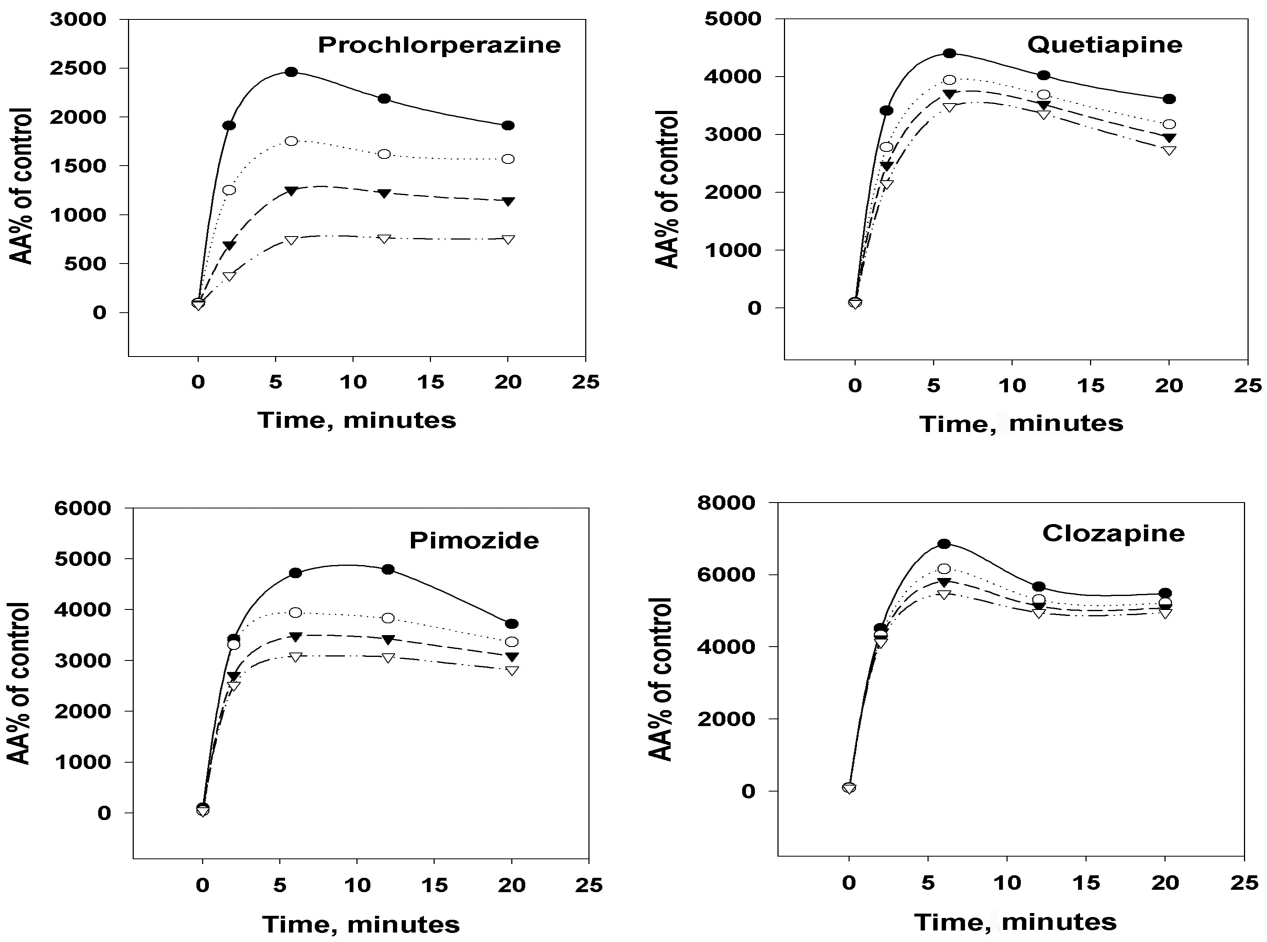

Figure 2 - Effects of prochlorperazine (PCP). quetiapine (QTP), clozapine (CLO), and pimozide (PMZ) on the time course of thrombin-induced [^{3}H]arachidonate liberation in platelets. [^{3}H]AA-prelabelled, gel-filtered platelets were incubated with 0.25 U/ml of thrombin at 37^{0}C in the absence (•) or presence of 10 (O), 20 (▼) or 30 (∇) μM of the drugs. At the times indicated aliquots were removed, extracted and the radioactivity of free [^{3}H]AA was determined as outlined in the methods section. Platelets from separate donors were used for each drug. The radioactivity of [^{3}H]AA is presented as percentage of that in the zero-time sample without drug or thrombin. Since PMZ and CLO were dissolved in dimethylsulfoxide (DMSO), appropriate amounts of this solvent were present in the control incubation mixture (0 μM drug); PCP and QTP were soluble in 0.15 M NaCl and appropriate amounts of the solvent were present in their control samples. AA - arachidonic acid.

the liberation of [^{3}H]AA with the same time course pattern, but to different degrees. The remaining 7 drugs gave similar time courses and inhibition patterns as the drugs shown in **Figure 2** when tested at 0 µM, 10 µM, 20 µM, and 30 µM with platelets from separate donors (data not shown). The comparison of the effects of all drugs on platelet PLA_2 was measured by liberation of [^{3}H]AA after incubation for 6 minutes with 0.25 U/ml of thrombin using platelets from 3 donors for all 11 drugs (30 µM) for each donor. The results are shown in **Figure 3**, and it should be pointed out that since some of the drugs were only soluble in DMSO and the others in 0.15 M NaCl, 2 controls with appropriate amounts of DMSO and 0.15 M NaCl, were included. Black bars in **Figure 3** represent these controls, and it can be seen that DMSO did not interfere with thrombin-induced liberation of [^{3}H]AA. **Figure 3** clearly shows that the phenothiazines CPZ, TFP, and PCP significantly reduced thrombin-induced platelet PLA_2 by 75-83% (relative to the solvent controls). The other typical psychotropic drug tested, HPD, only inhibited PLA_2 by 18% (**Figure 3**). The atypical drug CLO, did not reduce liberation of [^{3}H]AA significantly while OLP and QTP reduced the liberation by 8-18%; in contrast, PMZ reduced [^{3}H]AA release by 50% (**Figure 3**). The tricyclic antidepressants AMI and IMI gave approximately a 20% reduction while NTP gave a 70% reduction in the liberation of [^{3}H]AA (**Figure 3**).

Effects of the drugs on thrombin-induced phosphorylation of the 20 kDa MLC. [^{32}P]Pi-labelled platelets were incubated with or without 0.45 U/ml of thrombin in the presence of the drugs (30 µM) or their solvents for 6 minutes and the proteins separated by SDS-PAGE. **Figure 4** shows an autoradiogram of a representative gel where it is evident that the 20-kDa band is not labelled in the control platelets, but became labelled on incubation with thrombin without addition of the drugs. The degree of labelling, however, was weaker in the presence of CPZ, TFP, or PCP. The quantification of this labelling is discussed below. **Figure 4** also shows that a band around 47 kDa that was

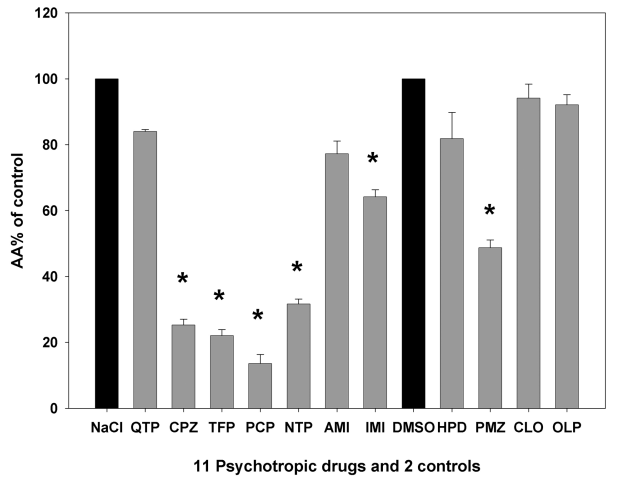

Figure 3 - Comparison of the effects of all 11 drugs investigated on thrombin-induced [^{3}H]arachidonate liberation in platelets. Gel-filtered, [^{3}H]AA-labelled platelets were incubated with 0.25 U/ml of thrombin at 37^{0}C in the absence (black bars) or presence of drugs (grey bars). After 90 seconds. the samples were extracted and processed as described in the methods section. The free [^{3}H]AA produced is presented as percentage of a control containing no thrombin or drugs (set to 100%). The experiment was performed with platelets from 3 different donors. The error bars (-) show the SEM values and the asterisks denote $p<0.05$ as compared to the controls without drug. AA - arachidonic cid, AMI - amitriptyline, CLO - clozapine; CPZ - chlorpromazine; DMSO - dimethylsulfoxide; HPD - haloperidol, IMI - imipramine; NTP - nortriptyline; OLP - olanzapine, PCP - prochlorperazine, PMZ - pimozide; QTP - quetiapine, TFP - trifluoperazine

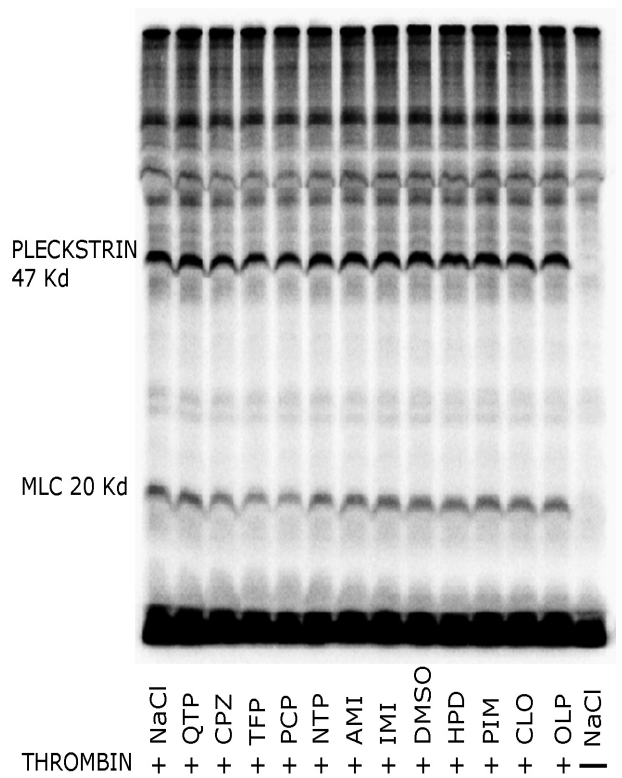

Figure 4 - SDS-PAGE of [^{32}P] Pi-labelled, gel-filtered platelets treated with thrombin in the absence or presence of drugs. [^{32}P]Pi-labelled, gel-filtered platelets were incubated as described in the legend to Figure 2, except that 0.45 U/ml of thrombin was used. After the reactions were stopped the protein samples were processed and subjected to SDS-PAGE electrophoresis as described in the methods section. The electropherogram was from platelets provided by one of the 3 donors whose myosin light chain phosphorylation is quantified in Figure 5. AMI - amitriptyline, CLO - clozapine; CPZ - chlorpromazine, DMSO - dimethylsulfoxide, HPD - haloperidol, IMI - imipramine, Kd - Kilo Dalton, MLC - myosin light chain, NTP - nortriptyline, OLP - olanzapine; PCP - prochlorperazine, PMZ - pimozide, QTP - quetiapine; TFP - trifluoperazine, NaCl - sodium chloride

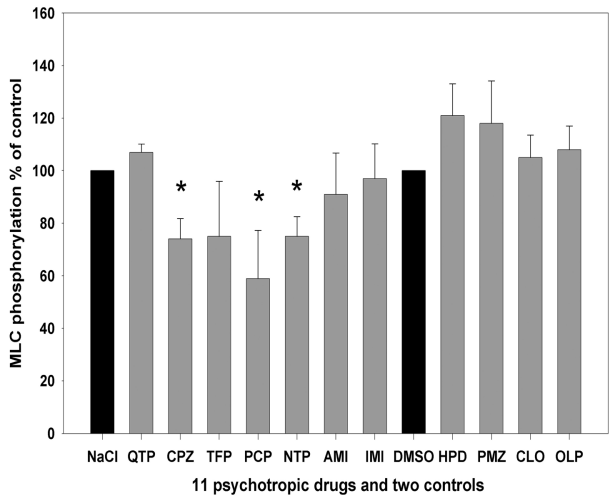

Figure 5 - Effect of all drugs on thrombin-induced myosin light chain phosphorylation. The data are from experiments with platelets from 3 different donors and performed as described in Figure 3. The error bars (-) show the SEM, and the asterisks denote $p<0.05$ as compared to the myosin light chain phosphorylation in the controls without drug. AMI - amitriptyline, CLO - clozapine; CPZ - chlorpromazine, DMSO - dimethylsulfoxide, HPD - haloperidol, IMI - imipramine, MLC - myosin light chain, NTP - nortriptyline, OLP - olanzapine, PCP - prochlorperazine, PMZ - pimozide, QTP - quetiapine, TFP - trifluoperazine, NaCl - sodium chloride.

not labelled in control platelets became heavily labelled when the cells were incubated with thrombin. This band represents pleckstrin, a substrate for protein kinase C that is activated by thrombin.[23] Chlorpromazine, TFP, and PCP reduced the thrombin-induced labelling of pleckstrin (**Figure 4**). The quantification of the level of 20 kDa MLC phosphorylation from the type of experiment shown in **Figure 4** is presented in **Figure 5**. Evidently, CLO, OLP, HPD, PMZ, QTP, AMI, and IMI stimulated or had no effect on MLC phosphorylation, while CPZ, TFP, PCP, and NTP reduced MLC phosphorylation by 20-30%.

Effects of the drugs on the surface pressure/mean molecular area relationships (isotherms) on monolayers of DPPC and DPPS. When DPPC monolayers were compressed on MilliQ water without drugs at 37^0C, there was no change in the surface pressure from 145 to 125-120 Å^2 (**Figure 6**, left panels, solid lines), indicative of the gaseous phase of the monolayers. Further compression caused a continuous, smooth rise in the surface pressure that started at 120-125 Å^2, the "lift-off", and rose to approximately 50 mN/m at 90 Å^2 where the curve became uneven, the "collapse point". Note that there are breakpoints in the curves at around 100 Å^2, indicating a structural change in the liquid-solid phase. The presence of 1 µM or 10 µM of IMI in the subphase caused negligible changes in the isotherm, while 1 µM and 10 µM NTP, AMI and OLP caused a slight shift of the isotherm to higher values of the mean molecular area (mma) (**Figure 6**, left panels). The isotherms for DPPS without drugs under the same conditions were somewhat different from those of DPPC (**Figure 6**, right panels, solid lines). The gaseous phase lasted over a larger mma region with a lift-off around 100-110 Å^2, the curves in the liquid-solid phase rose more sharply without breakpoints and the collapse occurred at higher surface pressures and lower mma. However, all drugs had much greater effects on the DPPS than on the DPPC monolayer isotherms with large increases in the mma where lift-off occurred and large increases in the mma were obtained in the 0-35 mN/m range (**Figure 6**, right panels). Of the 4 drugs shown in **Figure 6**, OLP had much less of an effect at 1µM than NTP, AMI, and IMI while the effects at 10 µM were similar for all 4 drugs.

Table 1 shows the percent change in mma relative to the control without drugs at lift-off, 10 mN/m, 20 mN/m, and 30 mN/m for all drugs tested on monolayers of DPPC and DPPS. All the drugs caused little change in the mma on DPPC monolayers, but markedly increased the mma of DPPS monolayers. However, there was little difference in this effect among the 11 drugs tested.

Discussion. Platelets are rich in cytosolic PLA_2, ($cPLA_2$) which is rapidly stimulated by treating the cells with thrombin or collagen through mechanisms involving translocation and phosphorylation of the enzyme and presence of cytosolic Ca^{2+}.[26-29] In the present study we demonstrate that, except for CLO, OLP, QTP, HPD, and AMI all the psychotropic drugs investigated significantly reduced the thrombin-induced AA liberation ($cPLA_2$ activity) in platelets with the following ranking order of increasing potency OLP<QTP<HPD<AMI<IMI<PMZ<NTP<CPZ<TFP<PCP. As far as we know, this is the first demonstration of inhibition of $cPLA_2$ in intact cells by QTP, OLP, HPD, PMZ, PCP and NTP. Amitriptyline and IMI have been reported to inhibit PLA_2 in brain slices, and TFP and CPZ inhibit $cPLA_2$ in thrombin-stimulated human platelets[10,29] and other cells.[30-32] There seems to be disagreement in the literature regarding the participation of the calmodulin/Ca^{2+}-dependent protein kinase in the activating mechanism of platelet $cPLA_2$.[21,26-28] We tested all psychotropic drugs for their possible effects on a calmodulin-dependent process in platelets, phosphorylation of the 20-kDa MLC chain by myosin light chain kinase (MLCK), under exactly the same conditions as used for measurement of PLA_2 (**Figures 4 & 5**). We found that CLO, OLP, QTP, TFP, AMI, and IMI had no significant effect on MLC

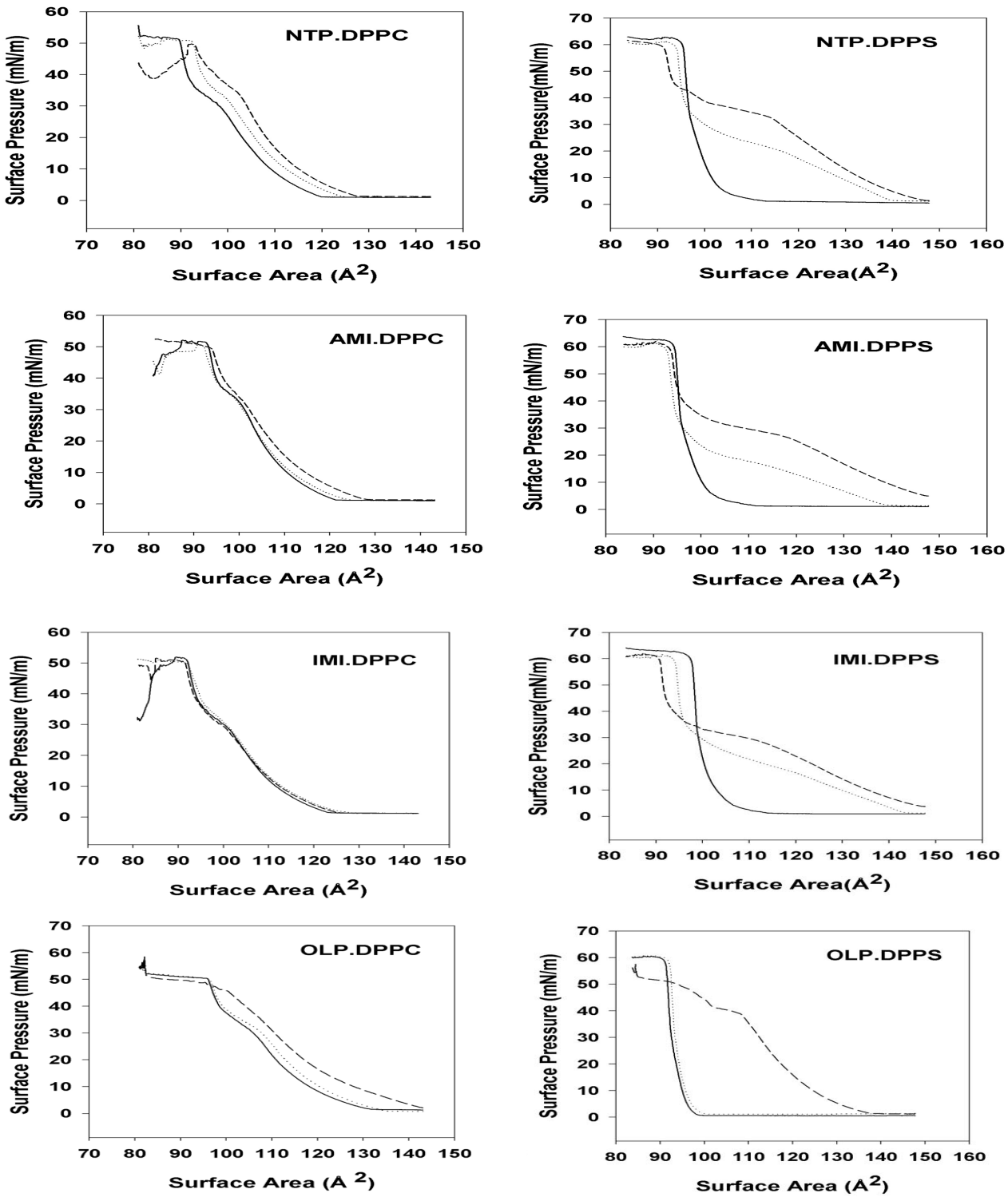

Figure 6 - Effects of imipramine (IMI), olanzapine (OLP), nortriptyline (NTP), and amitriptyline (AMI) on surface pressure/mean molecular area relations of 1,2 dipalmitoyl phosphatidylcholine (DPPC) and 1,2 dipalmitoyl phosphatidylserine (DPPS) monolayers. 1,2 dipalmitoyl phosphatidylcholine and DPPS monolayers on pure MilliQ water (—), 1 µM (.....) or 10 µM (-------) of the drugs (in MilliQ water) were compressed in the Langmuir apparatus at 37^{0}C and the surface pressure measured with a Wilhelmy plate.[8] Three experiments were performed for each drug, and gave identical results; the curves shown are from one of these experiments. A^2 - Angstrom square

Table 1 - Percentage change in mean molecular area (mma) relative to control (pure MilliQ water) at lift-off, 10, 20, and 30 mN/m caused by 10 μM of the amphiphilic drugs dissolved in MilliQ water. The experiments were performed as described in the legend to Figure 6, and the percentage change in mma at lift-off and surface pressures of 10, 20, and 30 mN/m was calculated. Appropriate amounts of dimethylsulfoxide (DMSO) were included in controls for pimozide, and clozapine.

Drugs	Phosphatidylcholine	Phosphatidylserine			
	20 mN/m	Lift-off	10 mN/m	20 mN/m	30 mN/m
Nortriptyline	6	44	30	25	23
Amitriptyline	2	41	40	16	13
Imipramine	2	33	31	17	9
Olanzapine	6	39	31	25	22
Clozapine*	3	48	36	25	18
Pimozide	7	48	56	42	23
Prochlorperazine*	3	63	27	20	16
Quetiapine*	3	45	48	27	14
Haloperidol*	4	52	41	30	23
Trifluoperazine*	12	41	31	27	21
Chlorpromazine†	7	53	30	26	19

* Values from our previous work.[6] †Values calculated from previous study.[7]

phosphorylation while HPD and PMZ actually stimulated this calmodulin-dependent enzyme. In contrast, CPZ, PCP, and NTP reduced MLC phosphorylation significantly by approximately 20%, and of these 3 drugs only CPZ has been reported to have calmodulin antagonist properties.[33] Thus, the inhibition of thrombin-induced $cPLA_2$ in platelets by CPZ, PCP, and NTP may involve inhibition of Ca^{2+}/calmodulin-dependent protein kinase. It should be noted that the phenothiazines have been found not to be specific calmodulin inhibitors, as they also interact with other Ca^{2+}-binding proteins.[20] One surprising finding when comparing the effects of the drugs on PLA_2 and MLCK in platelets, was the effect of PMZ. This diphenylbutylpiperidine inhibited PLA_2 by 50% (**Figure 3**) while it potentiated MLC phosphorylation by 20% (**Figure 5**), and therefore was unlikely to interfere with the PLA_2 activity through calmodulin antagonism. Both PMZ and HPD block Ca^{2+} channels,[34] and particularly, T-type channels.[35] Haloperidol stimulated MLCK more than PMZ, but only inhibited $cPLA_2$ by 15%. Just like HPD, PMZ does not inhibit PLA_2 through calmodulin antagonism. The brain contains several isoforms of PLA_2, both secretory and cytosolic isozymes, but these have not been reported to be inhibited by the psychotropic drugs studied here.[20] It is, however, possible that the different effects of the psychotropics on PLA_2 and MLCK are due to differences in subcellular localization of the 2 enzymes, as previously suggested for TFP.[10] Thus, the amphiphilic drugs studied have very strong affinity to the glycerophospholipid membranes (**Table 1**) and would be concentrated (first) in the platelet membranes in our experiments; these membranes both represent the substrate for PLA_2 and bind the enzyme during catalysis. The concentration of the amphiphiles in cytosol, where MLCK and MLC are located, would be far smaller than in the membrane, which could explain their different calmodulin antagonistic effects. The phenothiazines CPZ, TFP, and PCP, which were the strongest PLA_2 inhibitors in this study, also reduced the level of phosphorylation of the 47-kDa protein pleckstrin (**Figure 4**), which is the primary substrate for protein kinase C (PKC) in platelets.[23] As mentioned earlier, calmodulin antagonists also interact with proteins other than calmodulin, and CPZ and TFP have been reported to interact with protein kinase C-supported cellular processes.[36,37] The possibility therefore exists that PKC is involved in $cPLA_2$ activation in platelets and is inhibited by the phenothiazines as documented by their inhibition of both pleckstrin phosphorylation and PLA_2. Both pleckstrin and other phosphorylated proteins shown in **Figure 4** may have been affected by other drugs than the phenothiazines, which could not be quantified because the phosphorylation was too strong under the conditions used that were optimized to quantify phosphorylation of MLC. Human platelets contain mainly the $cPLA_2$ IVA ($cPLA_2\alpha$) isozyme,[38] that is activated by binding to phosphatidylinositol 4,5-

*bis*phosphate (PIP_2).[39] Chlorpromazine, TFP, PCP, CLO, QTP, and PMZ lower PIP_2 levels in thrombin-stimulated platelets,[6] and this lowering could contribute to the reduction of the $cPLA_2$ activity demonstrated in the present study. Haloperidol, on the other hand, caused an increase in PIP_2 levels in thrombin-stimulated platelets,[6] and it follows from **Figure 3** that HPD only slightly inhibited PLA_2. The inhibitory effects of the 11 drugs studied on platelet $cPLA_2$ may be due to, as discussed above, interference with the phosphorylation of its regulatory subunit,[19,35] its binding to PIP_2,[40] Ca^{2+}-dependent translocation,[19] and/or other regulatory processes. Both $cPLA_2$ and $sPLA_2$ catalyze hydrolysis that occurs on the water/membrane interface. Such interfacial catalysis has been thoroughly studied for $sPLA_2$,[41] and it has been made very clear that the rate of interfacial catalysis by $sPLA_2$ is greatly dependent on the structure of the organized lipid substrate. All the drugs studied markedly increased the mma of DPPS, but not of DPPC monolayers on a MilliQ water surface (**Table 1, Figure 6**). It should be noted that this is the first demonstration of this effect for CLO, IMI, NTP, AMI, PCP, and PMZ, and suggests, as discussed previously,[8] that the drugs intercalate among the DPPS molecules in the monolayer. However, this intercalating property, namely, increase in mma, has been maximized under the conditions used, ion-free water of pH around 6.5, which, as discussed elsewhere,[6] strongly promotes the mma-increasing effect of the negatively charged molecules of DPPS by cationic, amphiphilic molecules such as the drugs studied here. The mma-increasing effect on DPPS monolayers did not differ much among the drugs, whereas their effect on PLA_2 varied considerably. However, the latter effect was studied with platelets in a salt solution containing albumin at pH 7.4 and the platelet membranes contain many other phospholipids than DPPS, which may alter the intercalating effects of the drugs in very different ways. What we actually intended to demonstrate with the monolayer experiments was that all drugs studied are potentially membrane-active, which they indeed were. We therefore propose that the drugs intercalated to different degrees in the PS-containing inner leaflet of the platelet plasma membrane and alter the positioning of $cPLA_2$ to its substrates (so-called "alteration in substrate availability"),[39] which results in a decrease in the liberation of [^{3}H]AA. In this work we have compared the effects of 11 psychotropic drugs on $cPLA_2$ in platelets, and decided to test this at drug concentrations that gave the average maximal liberation of [^{3}H]arachidonate. The maximal effect for all drugs was found at 30 μM (with an incubation time with thrombin of 90 seconds), a concentration that is considerably higher than the effective serum concentrations (0.1-0.3 μM) reported in blood of patients during therapy. However, amphiphilic, psychotropic drugs partition between an aqueous milieu and biological membranes with partition coefficients ranging from 850 to 1700 and the partition depends both on temperature and on the length of the glycerophospholipid acyl chains and their degree of unsaturation.[42] The partition coefficient of CPZ between hexane and aqueous solutions, however, is in the range of 850-1700,[41] while the partition into liposomes containing PS has a coefficient in the order of 6.0-6.6 x 105,[42-45] and that of many other amphiphilic drugs may be even higher. Thus, it is likely that the drugs would exert inhibitory actions on PLA_2 under therapeutical circumstances in vivo. Liberation of AA by PLA_2 is the committed step in the synthesis of inflammatory eicosanoids, and inhibition of PLA_2 (feedback mechanism). Inhibition of eicosanoid synthesis by non-steroid anti-inflammatory drugs, such as acetylsalicylate, is widely used for prevention of diseases related to arterial thrombosis. Indeed, there are several reports on the counteraction of coronary ischemia by phenothiazines,[45-47] that could well be due to their strong inhibitory effect of PLA_2, as we have shown here. However, we are not aware of a systematic study of the hemostatic status of patients undergoing therapy with psychotropic drugs.

Limitations of the study. There are some limitations to the present study. Firstly, [^{3}H] AA may be incorporated into specific pools of phospholipids with sub-cellular locations (plasma membrane) that are particularly sensitive to the drugs studied. Secondly, platelets in other mammalian species like rats have a markedly different phospholipid composition than their neuronal cells,[48] which may influence the effects of the drugs on PLA_2 in different ways; thus the ranking order obtained for PLA_2 inhibition in platelets may be different from that in neuronal cells. Thirdly, our experiments were carried out in a closed system so that the results obtained may therefore not be directly applicable for an in vivo situation. Despite these limitations, our study clearly demonstrates potential novel effects of psychotropic/antidepressant drugs on cytosolic PLA_2. The overall results described in this paper extend our knowledge on the understanding of how psychotropic drugs exert an effect at the cellular level. It appears that inhibition of $cPLA_2$ is an important factor to achieve stability of the neurons. This is required to restore normal psychic function.

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