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Inhibitory Effects of Amitriptyline, Sertraline and Chlorpromazine on the Thrombin-induced Aggregation of Platelets

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ABSTRACT

Platelets resemble monoaminergic neurons in several respects, i.e. the uptake of 5-HT and its inhibition, the subcellular storage and release of 5-HT, and the metabolism of aromatic amines brought about by monoamine oxidase. And the 5-HT content of rabbit platelets is well known to be about 40 times higher than that of human platelets.

Therefore, this study was carried out to investigate the influences of amitriptyline (AMT) and sertraline (SRT) on the aggregation, contents of signaling second messengers, and protein phosphorylations of rabbit platelets in response to thrombin, 0.25 unit/ml, comparing with those of chlorpromazine (CPZ).

Thrombin-induced aggregation was inhibited by SRT (IC50: 4.37×10^{-5} M), CPZ (IC50: 5.76×10^{-5} M), and AMT (IC50: 1.15×10^{-4} M), respectively, and the aggregation by A23187 (1.0 μM) or PMA (320 nM) was also inhibited by SRT, CPZ, and AMT.

AMT, SRT, and CPZ had little affects on basal contents of platelet TXB_2 and PGE_2, but all of them inhibited the thrombin-induced increase of TXB_2.

Thrombin did not change the platelet contents of cAMP and cGMP. CPZ, AMT, and SRT produced the slight decrease of basal cAMP content, and their effects were not affected by thrombin-treatment. But SRT and AMT moderately increased the basal cGMP content, and the cGMP content of thrombin-stimulated platelets was gradually increased by the pretreatment with SRT, AMT, and CPZ. Particularly, the SRT-dependent increase of the cGMP content was notable. Platelet Ins(1,4,5)P_3 content was rapidly increased up to a plateau within 10 sec after thrombin-stimulation. AMT, SRT, and CPZ increased the basal Ins(1,4,5)P_3 content, and the thrombin-dependent increase was enhanced by pretreatment with CPZ and AMT, but was blunted by SRT.

Platelet $[Ca^{2+}]$ was rapidly increased up to a peak level within 20 sec after thrombin-stimulation. The increase of $[Ca^{2+}]i$ was significantly inhibited by AMT, SRT, and CPZ.

Thrombin- or PMA-induced phosphorylations of platelet 41~43 kDa and 20 kDa proteins were significantly inhibited by AMT, SRT, and CPZ.

These results suggest that the antiplatelet activities of AMT and CPZ may be considerably attributed to the inhibition of protein kinase C activity, and the activity of SRT may be associated with the inhibitory effect on the thrombin-induced increase of Ins(1,4,5)P_3 and the increasing effect on the cGMP content of platelets. Therefore, it seems to be evident that AMT and SRT may produce their antidepressant activity, at least, partly through the inhibition of protein kinase C activity or the increase of resting Ins(1,4,5)P_3 content and in case of SRT, to a lesser extent, via the increase of cGMP in the brain.

Key Words: Chlorpromazine, Amitriptyline, Sertraline, Platelet aggregation, Thromboxane, cAMP, cGMP, Ins(1,4,5)P_3, $[Ca^{2+}]$, Protein phosphorylation

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INTRODUCTION

Mood disorders are among the most common mental disorders in clinical practice, and depressive symptoms may occur in 13% to 20% of the population in the United States (Gold *et al.*, 1988). The biogenic monoamine hypothesis of depression has proposed that depression is caused by the reduction (or functional deficiency) of norepinephrine (Bunney and Davis, 1965) or 5-hydroxytryptamine (5-HT) (Gold *et al.*, 1988; Meltzer, 1990) at postsynaptic receptor sites. Most convincing evidence supporting the roles of monoamines in the pathogenesis of depression involves the proposed mechanisms of antidepressant drugs, which enhance the availability of these neurotransmitters at the postsynaptic receptor sites. However, the exact roles of monoamines in depression remain unclear. Nevertheless, 5-HT appears to be the most important neurotransmitter relevant to the pathophysiology of depression and the action of antidepressant drugs (Maes and Meltzer, 1995). And antidepressant drugs have shown to involve 5-HT receptors (Peroutka and Snyder, 1980; Kendall and Nahorski, 1985) and to be linked with adenylyl cyclase or phosphatidyl inositol signaling system (Nahorski *et al.*, 1986; Fisher and Arganoff, 1987).

Platelets also show the biologic resemblances with the monoaminergic neurons; uptake, storage, release and enzymatic metabolism of monoamine neurotransmitters, particularly 5-HT (Pletscher and Laubscher, 1980; Slotkin *et al.*, 1986), and have the well characterized metabolic pathways of signaling system, especially phosphoinositides turnover (Wilson *et al.*, 1987).

And the 5-HT content of rabbit platelets is well known to be about 40 times higher than that of human platelets (Da Prada *et al.*, 1980).

Therefore, the effects of two 5-HT reuptake inhibitors, amitriptyline (AMT) and sertraline (SRT) on the aggregation, changes of signal molecule metabolism, and protein phosphorylations of rabbit platelets in response to thrombin were studied in comparison with those of chlorpromazine, aiming to elucidate the possible modes of antidepressant actions.

EXPERIMENTAL PROCEDURES

Materials

Human plasma thrombin was obtained from Chrono-log. Prostaglandin E_1 (PGE_1) was a gift from Upjohn, and H-7, H-9 and ML-7 were purchased from Calbiochem. Phorbol 12-myristate 13-acetate (PMA), A23187, 3-isobutyl-1-methylxanthine (IBMX), and dibutyryl-cAMP (db-cAMP) were from Sigma. Aequorin was from Friday Harbor Photoproteins. [^{32}P]orthophosphate and radioimmunoassay systems for [^{3}H]cAMP, [^{3}H]cGMP, [^{3}H]inositol-1,4,5-trisphosphate ([^{3}H]Ins(1,4,5)P_3), [^{3}H]thromboxane B_2 ([^{3}H] TXB_2) and [^{125}I]prostaglandin E_2 ([^{125}I] PGE_2) were from Amersham.

Preparation of washed platelets

The washed platelets were prepared by the modification of Johnson's method (Johnson *et al.*, 1985). From rabbits under the anesthesia of ketamine (200 mg/kg, IM), blood was drawn via a carotid arterial cannula, directly mixed with 1/10 volume of 0.15 M trisodium citrate, and centrifuged at 2,000×g for 2 min at room temperature to yield platelet-rich plasma (PRP). PRP was added with 1 μM PGE_1, and then spun at 430×g for 10 min. The soft platelet pellet was suspended in 1 ml of HEPES-Tyrode's buffer (NaCl, 129 mM; $NaHCO_3$, 8.9 mM; KCl, 2.8 mM; KH_2PO_4, 0.8 mM; $MgCl_2$, 0.8 mM; dextrose, 5.6 mM; HEPES, 10 mM; pH 7.4) containing 10 mM EGTA and 1 μM PGE_1, at 0℃. The suspension was spun at 12,000×g at 4℃. The mushy pellet (pellet A) was suspended in 1 ml of HEPES-Tyrode's buffer without EGTA and PGE_1 (suspension B), and recentrifuged at 12,000×g, and this step was repeated one more time. The washed platelets were resuspended and diluted to 1.0 ($\pm$0.1)x10^8 platelets/ml HEPES-Tyrode's buffer, and the [Ca^{2+}] of the buffer was adjusted to 1 mM (suspension C).

Platelet aggregation

Suspension C was added with a dimethyl sulfoxide solution of CPZ, AMT, or SRT and incubated for 10 min at 37℃. The platelets

were stimulated by thrombin, A23187, or PMA, and monitored for 8 min by turbidimetric method (O'Brien, 1962).

Measurement of TXB_2

Thrombin-induced aggregation of platelets in suspension C was terminated at 0 sec and 2.5 min by adding 5 μl of 1 M citric acid (Marcus, 1990). The reaction medium was spun at 12,000 $\times$g for 5 min at 4℃, and the precipitate was applied to [^{3}H]TXB_2 assay system (Hart and Greenwald, 1979; Udenfriend *et al.*, 1985).

Measurement of PGE_2

Thrombin-induced aggregation of platelets in suspension C was terminated at 0 sec and 2.5 min by adding 500 μl of 80% ethanol and 10 μl of glacial acetic acid, and left at room temperature for 5 min. The PGE_2 obtained from platelets was quantitated using [^{125}I]PGE_2 assay system (Hart and Greenwald, 1979; Udenfriend *et al.*, 1985).

Measurement of cyclic nucleotides

Thrombin-induced aggregation of platelets in suspension C was terminated by adding 1 ml of ice-cold absolute ethanol prior to starting the reaction, and at 20, 45, and 120 sec after thrombin-stimulation. Ethanol-extracted cAMP and cGMP were dried under vacuum, and measured with [^{3}H]cAMP and [^{3}H]cGMP radioimmunoassay kits, respectively.

Measurement of Ins(1,4,5)P_3

The reaction was terminated by mixing the suspension C with 0.2 volume of ice-cold 20% (v/v) $HClO_4$ solution at 0, 6, 9, 15, 45 sec after thrombin-stimulation, and left at room temperature for 20 min to be settled down. The supernatant was adjusted to pH 7.2~7.5 with 1.5 M KOH containing 60 mM HEPES buffer and appropriate amount of phenol red solution (0.1% solution in 20% ethanol). After spinning, the Ins (1,4,5)P_3 of the supernatant was measured by [^{3}H]Ins (1,4,5)P_3 radioimmunoassay kit (Challiss *et al.*, 1988).

Measurement of platelet [Ca^{2+}]$_i$

Pellet A was resuspended in 280 μl of ice-cold HEPES-EGTA buffer A (NaCl, 150 mM; HEPES, 5 mM; ATP, 5 mM; $MgCl_2$, 2 mM; EGTA, 10 mM; PGE_1, 1 μM; pH 7.0), and mixed with 20 μl of the reconstituted aequorin (1 mg of lyophilized aequorin in 333 μl of 7~10 mM EGTA solution; Blinks *et al.*, 1976). Platelets were incubated for 1 hour over melting ice, then spun at 12,000$\times$g at 4℃, and the pellet was resuspended in 1 ml of HEPES-EGTA buffer B (NaCl, 150 mM; HEPES, 5 mM; ATP, 5 mM; $MgCl_2$, 10 mM; EGTA, 0.1 mM; PGE_1, 1 μM; pH 7.0) and incubated for 1 hour over melting ice. The platelets loaded with aequorin were recalcified three times by adding 1 μl aliquots of 100 mM $CaCl_2$ with 5 min interval. The suspension was layered onto a Sepharose 2B gel column (bed volume of 9 ml) pre-equilibrated with HEPES-Tyrode's buffer containing 1 mM $CaCl_2$ and 0.1% bovine serum albumin. The increase of platelet [Ca^{2+}]$_i$ in response to thrombin was monitored by luminescence of intracellular Ca^{2+}-aequorin complex in platelets (Johnson *et al.*, 1985).

Analysis of protein phosphorylation

Platelets of suspension B (1 ml) were incubated for 2 hours with 200 μCi of [^{32}P]Pi (HCl free) and spun upto 12,000$\times$g, and the precipitate was washed by resuspending in HEPES-Tyrode's buffer and spun again. The pellet of [^{32}P] Pi-incorporated platelets was finally resuspended and diluted to 1.0 ($\pm$0.1)$\times 10^9$ platelets/ml in HEPES-Tyrode's buffer, and the [Ca^{2+}] of the suspension was adjusted to 1 mM. Each batch of the platelet suspension was incubated with CPZ, AMT, SRT, H-7, H-9, ML-7 and db-cAMP for 10 or 20 min at 37℃. Platelets were stimulated by thrombin or PMA for 3 min and solubilized in the 186 mM Tris-HCl buffer (pH 6.75) containing 15% glycerol, 6% 2-mercaptoethanol, 9% SDS, and 0.002% bromophenol blue, and boiled in water for 5 min. The solubilized proteins were separated by the SDS/10~15% polyacrylamide gel (Laemmli, 1970; Feinstein *et al.*, 1983) and stained with Coomassie Brilliant Blue R, and then autoradiographed on Kodak X-OMAT AR film at −70℃.

Table 1. IC50 of chlorpromazine, amitiriptyline, and sertraline for the platelet aggregation induced by thrombin (0.25 unit/ml), A23187(1.0 μM), and PMA(320 nM).

	Thrombin	A23187	PMA
Chlorpromazine	5.76×10^{-5} M	8.34×10^{-5} M	9.06×10^{-6} M
Amitriptyline	1.15×10^{-4} M	1.75×10^{-4} M	1.48×10^{-5} M
Sertraline	4.37×10^{-5} M	1.21×10^{-4} M	1.05×10^{-5} M

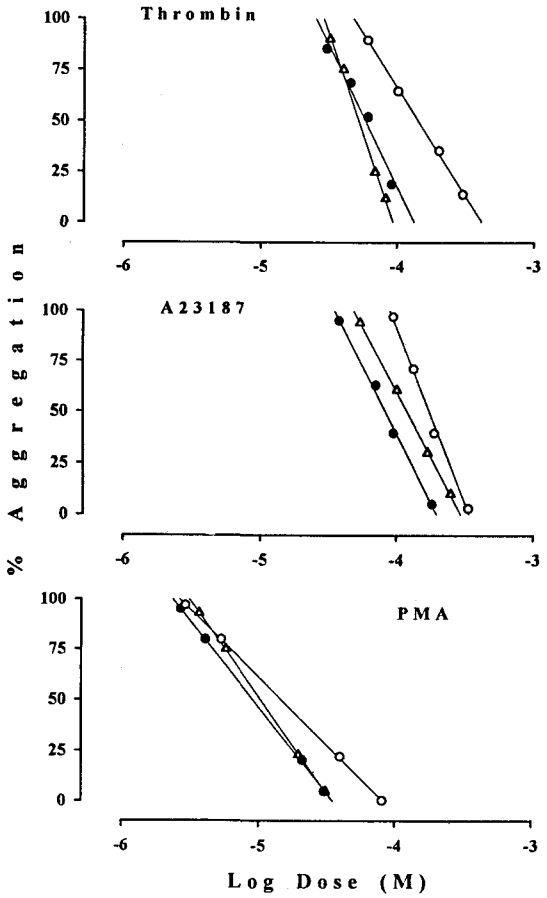

Fig. 1. Inhibitory effects of chlorpromazine(●), amitriptyline(○), and sertraline(△) on the platelet aggregation induced by thrombin (0.25 unit/ml), A23187 (1.0 μM), and PMA (320 nM).

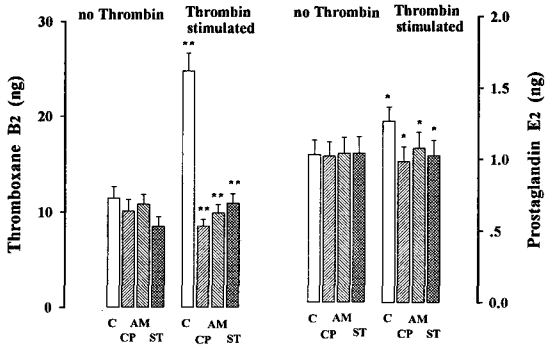

Fig. 2. Effects of chlorpromazine, amitriptyline, and sertraline on the thromboxane B_2 and prostaglandin E_2 contents in the platelets stimulated by thrombin for 2.5 minutes.
Abbreviations
C: control
CP: chlorpromazine, 1×10^{-4} M
AM: amitriptyline, 1.5×10^{-4} M
ST: sertraline, 1×10^{-4} M
* and ** indicate $p<0.05$ and $p<0.01$, respectively.

RESULTS

Inhibition of platelet aggregation

Thrombin (0.25 unit/ml) induced the typical biphasic aggregation of platelets to 80±5% light transmission of HEPES-Tyrode's buffer, and it was the intermediate dose to initiate shape change, primary wave and the irreversible secondary wave (Harlan and Harker, 1981). Other agonists, A23187 and PMA (1.0 μM and 320 nM, respectively), also induced platelet aggregation.

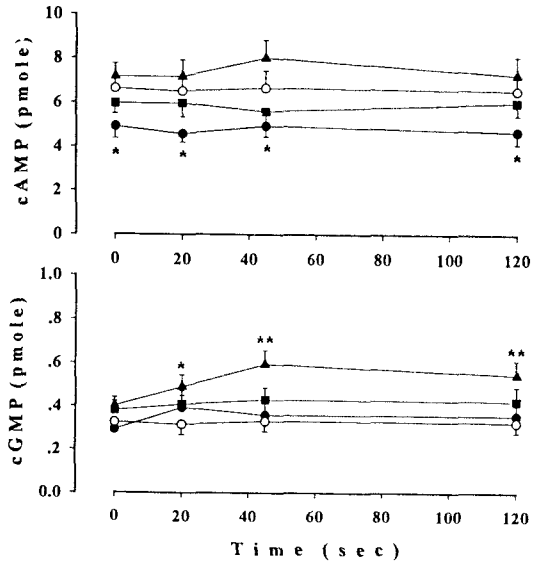

Fig. 3. Effect of chlorpromazine on the cAMP and cGMP contents in thrombin-stimulated platelets.
○: control, DMSO 0.1% ▲: 3×10^{-5} M
■: 1×10^{-4} M ●: 2×10^{-4} M
* and ** indicate $p<0.05$ and $p<0.01$, respectively.

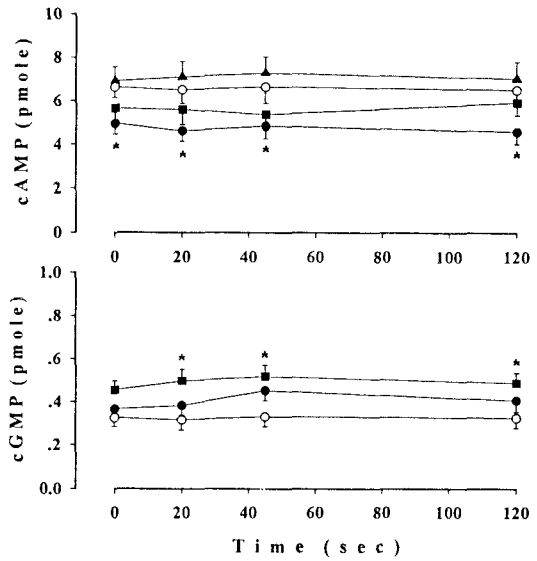

Fig. 4. Effect of amitriptyline on the cAMP and cGMP contents in thrombin-stiumlated platelets.
○: control, DMSO 0.1% ▲: 6×10^{-5} M
■: 1×10^{-4} M ●: 3×10^{-4} M
* and ** indicate $p<0.05$ and $p<0.01$, respectively.

The inhibitory effects of CPZ, SRT, and AMT on the platelet aggregation were compared (Fig. 1, Table 1). IC50 of the drugs to PMA-induced aggregation were 10 times less than those to thrombin- and A23187-induced.

Changes of platelet TXB_2 and PGE_2 contents

CPZ (1×10^{-4} M), AMT (1.5×10^{-4} M), and SRT (1×10^{-4} M) had no effects on the basal content of TXB_2, the stable metabolite of TXA_2 (Hamberg and Samuelsson, 1974), and that of PGE_2 in platelets (Fig. 2).

Fig. 2 (left) showed that TXB_2 generation was significantly increased by thrombin; it was increased to 216.6% of basal level at 2.5 min after stimulation. All three drugs nearly abolished this increase of TXB_2. Increase of PGE_2 by thrombin, 23.1%, was also inhibited by the drugs as shown in Fig. 2 (right).

Changes of platelet cyclic nucleotides contents

cAMP content in resting platelets was not changed by thrombin-stimulation through the sequence of 20, 45, and 120 sec after stimulation (Fig. 3~5, upper). Resting cGMP content was not also influenced after thrombin-stimulation of 20, 45, and 120 sec (Fig. 3~5, lower).

3×10^{-5} M CPZ showed a little increase of cAMP before and after thrombin-stimulation. And 2×10^{-4} M decreased cAMP of resting and thrombin-stimulated platelets. 1×10^{-4} M had a tendency of decreasing the cAMP but it was not remarkable (Fig. 3-upper). In spite of little changes of resting cGMP by doses of CPZ, the smallest dose of 3×10^{-5} M increased the cGMP of thrombin-stimulated platelets. And 1×10^{-4} M and 2×10^{-4} M of CPZ had no effects on cGMP of stimulated platelets as well as the content of resting (Fig. 3-lower).

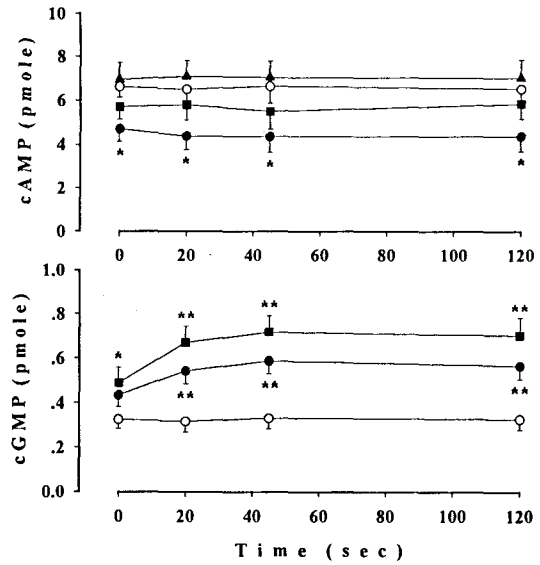

Fig. 5. Effect of sertraline on the cAMP and cGMP contents in thrombin-stimulated platelets.
○: control, DMSO 0.1% ▲: 3×10^{-5} M
■: 1×10^{-4} M ●: 2×10^{-4} M
* and ** indicate $p<0.05$ and $p<0.01$, respectively.

3×10^{-4} M AMT decreased the cAMP and 1×10^{-4} M showed a little decrease of cAMP (Fig. 4-upper). 1×10^{-4} M AMT increased cGMP in resting and thrombin-stimulated platelets and higher dose, 3×10^{-4} M, showed a little increase of cGMP (Fig. 4-lower).

Like CPZ and AMT, SRT decreased the basal and stimulated cAMP by 2×10^{-4} M, and 1×10^{-4} M and 3×10^{-5} M SRT had no significant effects on the cAMP (Fig. 5-upper). SRT, 1×10^{-4} M and 2×10^{-4} M, increased the cGMP in resting platelets, and the increase was enhanced by thrombin-stimulation (Fig. 5-lower). The increase of cGMP by 1×10^{-4} M SRT was higher than the increase by 2×10^{-4} M.

Changes of platelet Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ levels

Within 10 sec after thrombin stimulation, Ins (1,4,5)P_3 formation began to rise up to a plateau (Fig. 6). CPZ (1×10^{-4} M) and AMT (1.5×10^{-4} M) increased resting content of Ins (1,4,5)P_3

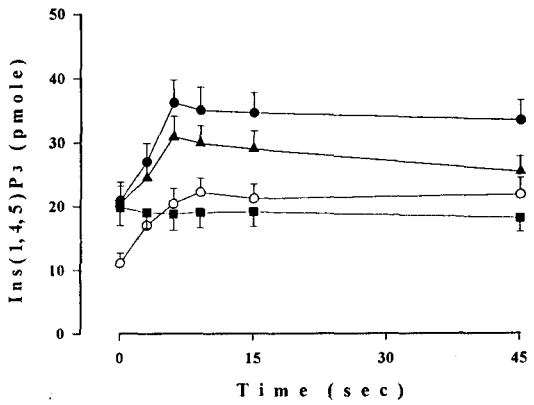

Fig. 6. Effects of chlorpromazine (1×10^{-4} M; ●), amitriptyline (1.5×10^{-4} M; ▲), and sertraline (1×10^{-4} M; ■) on the Ins(1,4,5)P_3 content in thrombin-stimulated platelets.
○: control, DMSO 0.1%

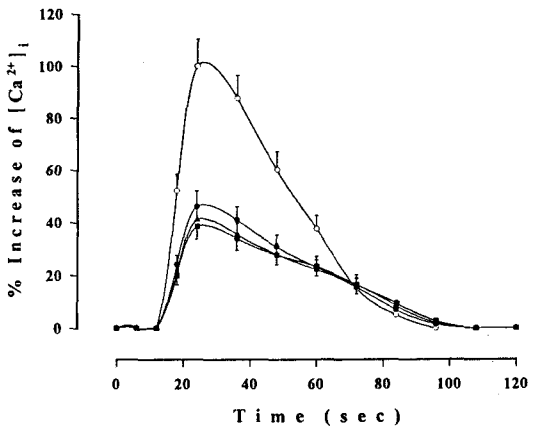

Fig. 7. Effects of chlorpromazine (1×10^{-4} M; ●), amitriptyline (1.5×10^{-4} M; ▲), and sertraline (1×10^{-4} M; ■) on the increase of $[Ca^{2+}]_i$ in thrombin-stimulated platelets.
○: control, DMSO 0.1%

and thrombin enhanced the increase of Ins (1,4,5)P_3. SRT (1×10^{-4} M) increased the basal content but further increase of Ins (1,4,5)P_3 after thrombin stimulation was not shown.

The effects of two antidepressants and CPZ

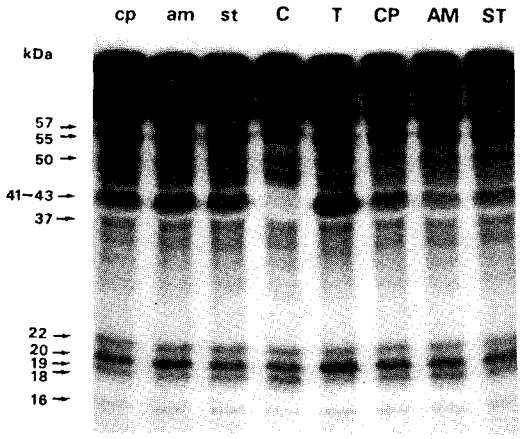

Fig. 8. Effects of chlorpromazine, amitriptyline, and sertraline on the thrombin-induced phosphorylations of platelet proteins.
Abbreviations
C: control, DMSO 0.1%
T: thrombin, 0.25 unit/ml
cp & CP: chlorpromazine, 5.76×10^{-5} M & 1.2×10^{-4} M
am & AM: amitriptyline, 1.15×10^{-4} M & 2.3×10^{-4} M
st & ST: sertraline, 4.37×10^{-5} M & 8.7×10^{-5}M

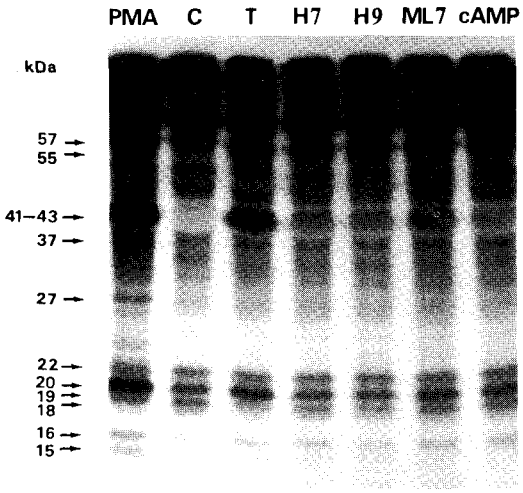

Fig. 9. Effects of H-7, H-9, ML-7, and db-cAMP on the thrombin-induced phosphorylations of platelet proteins.
Abbreviations
C: control, DMSO 0.1%
T: thrombin, 0.25 unit/ml
PMA: 320 nM H7: 5×10^{-5} M
H9: 5×10^{-5} M ML7: 5×10^{-5} M
cAMP: db-cAMP, 7.5×10^{-4} M

on mediating intracellular Ca^{2-} signaling were examined in aequorin-loaded platelets (Fig. 7). Increase of $[Ca^{2+}]_i$ followed the Ins (1,4,5)P_3 rise and reached to a peak within 20 sec after thrombin-stimulation. On getting to peak, $[Ca^{2+}]_i$ gradually decreased. And this increase was significantly reduced to less than 40% by AMT (1.5×10^{-4} M) and SRT (1×10^{-4} M), and to about 50% by CPZ (1×10^{-4} M).

Phosphorylation of platelet proteins

After 2 hours incorporation of [^{32}P]orthophosphate, platelet phosphoproteins, of pre- and 3 min post-stimulation by thrombin, were compared on the SDS/10~15% polyacrylamide gel electrophoresis.

Platelets represented the phosphorylations of 57 kDa, 50 kDa, 47 kDa, 22 kDa, 20 kDa and 19 kDa proteins. Thrombin profoundly phosphorylated 41~43 kDa and 55 kDa proteins, and increased the phosphorylations of 37 kDa, 20 kDa and 16 kDa proteins. But phosphorylations of 57 kDa and 19 kDa proteins were inhibited by thrombin (Fig. 8, 9).

Of the enhanced phosphorylations by thrombin, CPZ (5.76×10^{-5} M, 1.2×10^{-4} M), AMT (1.15×10^{-4} M, 2.3×10^{-4} M) and SRT (4.37×10^{-5} M, 8.7×10^{-5} M) inhibited the phosphorylations of 41~43 kDa and 37 kDa proteins in dose-dependent mode, and the phosphorylation of 20 kDa protein was also inhibited by the drugs (Fig. 8).

db-cAMP (7.5×10^{-4} M) inhibited phosphorylation of 41~43 kDa protein due to thrombin, and reversed the thrombin-inhibited phosphorylations of 34 kDa and 19 kDa proteins, and also increased slightly 22 kDa protein phosphorylation. But it could not inhibit the 22 kDa protein phosphorylation by thrombin (Fig. 9).

Of inhibitors of protein kinases, H-7 (5×10^{-5} M) and H-9 (5×10^{-5} M) inhibited the phosphorylations of 41~43 kDa and 20 kDa proteins, and reversed the inhibited 34 kDa and

19 kDa protein phosphorylations. And ML-7 (5×10^{-5} M), more specific for myosin light-chain kinase, reversed the inhibited phosphorylations of 34 kDa and 19 kDa proteins, and slightly increased 22 kDa protein phosphorylation, like db-cAMP. But it showed little effect on the phosphorylations of 41~43 kDa and 20 kDa proteins (Fig. 9).

DISCUSSION

Most convincing evidence supporting the role of monoamines in pathogenesis of depression involves the proposed mechanism of antidepressant drugs, which enhance monoaminergic neurotransmission in the brain. Although monoamines, particularly 5-HT, appears to be the most important neurotransmitter relevant to the pathophysiology of depression and the action of antidepressant drugs (Maes and Meltzer, 1995; Peroutka and Snyder, 1980; Kendall and Nahorski, 1985), the exact therapeutic mechanism of antidepressant drugs has been remained to be elucidated.

However, it has been observed that tricyclic antidepressants and phenothizines altered the activities of phospholipase C or phosphatases (Osborne, 1988; Pandey *et al.*, 1991) and chronic treatment with antidepressants causes a decrease in α- and β-adrenoceptors, 5-HT_2 receptors, and receptor mediated formation of cAMP in the rat brain (Sulser, 1979; Perouka and Snyder, 1980; Smith *et al.*, 1981).

Several tissues, including brain (Consolo *et al.*, 1988; Supattapone *et al.*, 1988) and platelets (Jakobs *et al.*, 1986), show the inverse relation between phosphoinositide system and cAMP second messenger system in the signal transduction.

And Shimizu *et al.*[1,2] (1993) reported that high concentrations of tricyclic antidepressant drugs mobilize Ca^{2+} from Ins(1,4,5)P_3-sensitive Ca^{2+}-storages of primary cultured neurons.

It has been well known that platelets show the biologic resemblances with the monoaminergic neurons in the kinetics of monoamine neurotransmitters (Pletscher and Laubscher, 1980; Slotkin *et al.*, 1986), and platelets also have the well characterized signaling systems, especially phosphoinositide turnover (Wilson *et al.*, 1987).

So, in order to elucidate a possible mechanism of antidepressant drug action, this study invetigated the effects of AMT and SRT on the changes of several signal molecules and the protein phosphorylations of rabbit platelets in response to thrombin.

Thrombin-induced aggregation of platelets is known to be occurred with the release of arachidonic acid and its cyclooxygenase metabolites (Colard *et al.*, 1986). The mechanism of arachidonic acid release is likely inferred to involve the activation of phospholipase A_2 due to the increase of $[Ca^{2+}]_i$ (Siess *et al.*, 1985; Pollock *et al.*, 1984), with the release of arachidonic acid mostly bound to platelet membrane phospholipids (Derksen and Cohen, 1975; Bills *et al.*, 1976). Thromboxanes transformed from released arachidonic acid are known to interact with specific platelet membrane receptors that are coupled to further activation of the phosphoinositide cycle and to the increase of $[Ca^{2+}]_i$ (Siess *et al.*, 1985; Pollock *et al.*, 1984). Thus the inhibition of thromboxane generation by the CPZ, AMT, and SRT may be responsible for the inhibition of amplification of platelet activation as well as the inhibitory index of platelet response to thrombin stimulation. PGE_2 is one of the inhibitory modulators of platelet aggregation through increasing the cAMP content, and its generation in platelets by thrombin is less than the generation of thromboxanes. Local anesthetics (Feinstein *et al.*, 1977) and propranolol (Vanderhoek and Feinstein, 1979) have been proposed to inhibit arachidonic acid release, and phospholipase A_2 is one of the suggestive targets of them. And inhibition of phospholipase A_2 activity by CPZ is indirectly evidenced (Ishigooka *et al.*, 1985). Almost complete inhibition of thrombin-induced TXB_2 and PGE_2 generation by CPZ, AMT, and SRT proposes the possibility that these drugs may inhibit phospholipase A_2 activity directly or through the inhibitory effects on the increase of $[Ca^{2+}]_i$. By the inhibitory effects on protein kinase C activation, Ca^{2+} mobilization, fibrinogen receptor exposure, myosin light chain phosphorylation, actin polymerization, and

cytoskeletal assembly, cAMP has been known as the inhibitory signal molecule to platelet functions (Siess, 1989).

Activation of platelets by thrombin had no effects on the metabolism of cGMP as well as cAMP. It is unlikely that the activation of platelets is related to the cyclic nucleotide system, in contrast to the inhibitory process of platelets. Unlike thrombin, CPZ, AMT, and SRT (3×10^{-5} M to 3×10^{-4} M) supposed to have effects on adnylyl-/guanylyl-cyclase or phosphodiesterases activities. The dose-dependent effect of decreasing cAMP content suggested the possibility that the high concentrations of CPZ, AMT, and SRT inhibited the adenylyl cyclase activity, but the inhibition of enzyme activity seemed not to be correlated with the antiaggregatory activities. Further increase of cGMP content by CPZ, AMT, and SRT at doses of 3×10^{-5} M and 1×10^{-4} M may present a clue of the role of this molecule in the pharmacotherapeutic activities of the drugs.

Tricyclic antidepressants stimulated basal formation of inositol phosphates but decreased agonist-stimulated formation of inositol phosphates in rat cortical slices (Osborne, 1988). CPZ was also found to increase the accumulation of inositol phospholipids in rat and guinea pig cortical slices (Hokin-Neaverson, 1980; Pappu and Hauser, 1981) and to decrease the formation of Ins(1,4,5)P_3 in human platelets most likely due to the decrease of phosphatidylinositol 4,5-bisphosphate pool (Strunecka *et al.*, 1987). Shimizu *et al.*[1] (1993) suggested that high concentrations (10^{-4} M to 10^{-3} M) of antidepressants, especially AMT, induced Ins(1,4,5)P_3 production and a consequent Ca^{2+} release from Ins(1,4,5)P_3-sensitive Ca^{2+} pools through direct or indirect activation of phospholipase C in rat frontocortical neurons. In human platelets, AMT and CPZ inhibited thrombin-stimulated formation of inositol 1,4-bisphosphate and Ins(1,4,5)P_3 but had no effect on inositol 1-phosphate formation. These inhibitory effects on inositol phosphates production may be due to the inhibition of phospholipase C by antidepressants and the activation of phosphomonoesterase and /or inhibition of phospholipase C by phenothiazines (Pandey *et al.*, 1991). It has been also reported that CPZ enhanced Ins(1,4,5)P_3 accumulation in thrombin-stimulated platelets, indicating possible inhibition of phosphatases (Wakatabe *et al.*, 1991). Therefore, in cortical neurons and platelets, agonists such as thrombin certainly stimulate the production of Ins(1,4,5)P_3, but effects of antidepressants and CPZ on the basal and stimulated production of Ins(1,4,5)P_3 have remained to be equivocal.

The change of Ins(1,4,5)P_3 production alone may represent the alteration of enzyme activities of phospholipase C and inositol phosphates phosphatases. Our data clearly showed that CPZ (2×10^{-4} M) and AMT (3×10^{-4} M) increased basal content and enhanced the rise of Ins(1,4,5)P_3 content due to thrombin. SRT (2×10^{-4} M) significantly increased the basal Ins(1,4,5)P_3 content in spite of no effect on the thrombin-stimulated increase. The possibility emerges that CPZ and AMT of high concentrations inhibit phosphatase that metabolizes the inositol phosphates.

In cultured rat frontocortical neurons loaded with fura-2, high concentrations of antidepressants, especially AMT (10^{-4} M to 10^{-3} M), elicited transient increase in $[Ca^{2+}]_i$ in dose-dependent manner mainly from Ins(1,4,5)P_3-sensitive Ca^{2+} pool (Shimizu *et al.*[2], 1993). In this study with aequorin-loaded platelets, 10 min incubation with CPZ, AMT, and SRT inhibited the increase of aequorin-Ca^{2+} signal due to thrombin. This evidence of inhibition of Ca^{2+} mobilization may be pertinent to the inhibition of aggregation and the suggested inhibition of phospholipase A_2 activity. But the amplification of thrombin-stimulated increase of Ins(1,4,5)P_3 content by CPZ and AMT corresponds to the report that AMT is the possible Ins(1,4,5)P_3-sensitive Ca^{2+} mobilizing agent (Shimizu *et al.*[1], 1993). Two possibilities cannot be excluded. It is unlikely that $[Ca^{2+}]_i$ increase in thrombin-stimulated platelets depends entirely on the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. Also during the incubation period (10 min) with CPZ, AMT, and SRT which was enough to increase basal Ins(1,4,5)P_3 content, parts of aequorin loaded in platelets might be consumed by mobilized Ca^{2+} due to the drugs, before thrombin stimulation.

Parts of the basal phosphorylations of platelet proteins in this study might be induced by the addition of PGE_1 during platelet prepara-

tion. And db-cAMP increased 22 kDa protein phosphorylation. 22 kDa phosphoprotein has been known as thrombolamban whose phosphorylation is known to be modulated by cAMP and associated with the activation of Ca^{2+}-ATPase (Fox *et al.*, 1987; Corvazier *et al.*, 1992). But CPZ, AMT, and SRT showed no effects on this phosphoprotein.

41~43 kDa protein, phosphorylated by thrombin and PMA in this study, has been evidenced to be the protein kinase C activity (Tyers *et al.*, 1988; King and Rittenhouse, 1989). 20 kDa of myosin light chain has been known to be phosphorylated by both myosin light chain kinase and protein kinase C (Ikebe *et al.*, 1987; Higashihara *et al.*, 1991).

The result that thrombin-induced phosphorylation of 41~43 kDa protein was dose-dependently inhibited by CPZ, AMT, and SRT, which was comparable with the inhibition by H-7, and H-9, clearly showed the inhibition of protein kinase C activity by the drugs. Additionally, the enhanced phosphorylation of 20 kDa protein due to thrombin was inhibited by CPZ, AMT, SRT, H-7, and H-9, but db-cAMP had no effect on this phosphorylation. In addition to protein kinase C inhibition, the inhibition of 20 kDa protein phosphorylation by CPZ, AMT, and SRT might be also responsible to a part of antiaggregatory activities of the drugs.

CPZ and local anesthetics such as dibucaine and tetracaine were suggested to inhibit protein kinase C activity through the interaction with phospholipid, phosphatidylserine (Mori *et al.*, 1980; Schatzman *et al.*, 1981). It is possible that CPZ, AMT, and SRT inhibit the factors such as DAG, Ca^{2+}, or phosphatidylserine of protein kinase C activation (Mori *et al*, 1980).

These results suggest that the antidepressant effects of AMT and SRT may be attributed partly to the inhibition of protein kinase C activity and the increase of resting Ins(1,4,5)P_3 level, and in case of SRT, to a lesser extent, to the increase of stimulated formation of cGMP in the brain.

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=국문초록=

Thrombin성 혈소판응집에 대한 Amitriptyline, Sertraline 및 Chlorpromazine의 억제작용

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혈소판은 혈전기전의 중요요소로, monoamine성 신경전달물질의 대사에 있어서 신경계와 유사점을 가지고 있다. 따라서 항우울약물인 amitriptyline (AMT)과 sertraline (SRT)의 혈소판응집 억제와 이에 의한 세포내 신호전달 물질의 함량변동 및 단백인산화에 대한 영향을 chlorpromazine (CPZ)과 비교연구함으로써, 이들 약물의 혈소판응집 억제작용의 효능을 검정하고, 항혈소판 및 항우울 작용기전의 일단을 규명하고자 하였다.

SRT, CPZ 및 AMT은 thrombin (0.25 unit/ml)에 의한 혈소판응집을 억제하였으며,각각의 IC50은 4.37×10^{-5} M, 5.76×10^{-5} M 및 1.15×10^{-4} M이었다. 이러한 억제효과는 A23187 (1.0 μM)및 PMA (320 nM)에 의한 혈소판응집에 대해서도 유사하게 나타났다. thrombin은 혈소판응집과 아울러 thromboxane B_2 및 prostaglandin E_2 생성을 유의하게 증가시켰으며, 이러한 arachidonic acid 생성은 CPZ, AMT 및 SRT에 의하여 현저하게 억제되었다.

CPZ, AMT 및 SRT은 cAMP 함량을 용량의존적으로 감소시켰으며, SRT, AMT (1×10^{-4} M) 및 CPZ (3×10^{-5} M)은 cGMP 함량을 증가시키는 경향을 보였다.

한편, Ins(1,4,5)P_3 함량은 thrombin 부하 후 10초 이내에 정점에 도달한 후 45초 이후까지 유지된다. CPZ과 AMT은 혈소판의 Ins(1,4,5)P_3 함량을 현저히 증가시키며, thrombin에 의한 증가도 유의하게 증강시킨다. SRT은 혈소판의 Ins(1,4,5)P_3을 증가시키나, thrombin 부하 후 증강되지는 않았다.

Ins(1,4,5)P_3 증가에 이어서, $[Ca^{2+}]_i$은 thrombin 부하 후 20초에 최고점에 이르며, 이러한 $[Ca^{2+}]_i$ 증가는 세 약물에 의하여 현저하게 억제되었다.

혈소판 단백인산화에 대해서, thrombin은 41~43 kDa 및 20 kDa 단백인산화를 현저하게 증가시켰으며, 이는 AMT, SRT 및 CPZ에 의하여 억제되었다.

CPZ, AMT 및 SRT 등의 세 약물은 유의한 항응집효과와 thromboxane생성억제 효과를 나타냈으며, 이들 약물에 의한 protein kinase C 활성억제 및 Ins(1,4,5)P_3의 함량증가는 각각 이들약물의 항응집효과 및 항우울성 작용기전과 연관될 수 있음을 시사한다.