Inhibition of Phospholipase A2 by Tiaramide in Rabbit Platelets

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Abstract—The mechanism by which tiaramide inhibited platelet aggregation was investigated using phospholipid labelling techniques by 14C-arachidonic acid (AA) and thin-layer chromatography. Tiaramide did not affect cyclo-oxygenase nor thromboxane synthetase, because TXB2 was detected in tiaramide-treated platelets, unlike aspirin-treated ones, and PGE2 and PGD2 did not increase, unlike in platelets treated with OKY-1581 (an inhibitor of thromboxane synthetase). Total phospholipid radioactivity was 82.5% of radioactivity recovered before aggregation, and this decreased to 49.0% (n=5, P<0.05) after aggregation by collagen (30 μg/ml). AA radioactivity was 9.6% before aggregation and 40.0% after. Tiaramide (100 μM) restored total phospholipid and AA levels to those before aggregation. Tiaramide decreased the amount of AA liberated from 2-(3H-arachidonyl)phosphatidylcholine by whole platelet phospholipase A2 (PLA2). Tiaramide at 10 μM inhibited collagen-induced aggregation, but not that by AA. Tiaramide did not affect 45Ca-uptake by itself nor collagen-induced 45Ca-uptake from the external medium. Tiaramide did not inhibit intracellular Ca mobilization, and it did not affect the calmodulin-dependent cyclic nucleotide phosphodiesterase of rabbit brain. These facts suggest that tiaramide inhibits platelet PLA2 through mechanisms other than the blockade of Ca-influx and intracellular Ca mobilization or antagonism to calmodulin.

Tiaramide, a nonsteroidal anti-inflammatory drug, is a basic compound, not acidic like aspirin. Our previous reports (1, 2) showed that tiaramide inhibited platelet aggregation produced by ADP or collagen and decrease of platelet electrophoretic mobility produced by each of them, and it reduced cyclic AMP levels of rabbit platelets, whereas aspirin and indomethacin did not. These facts indicate that there are some differences in mechanism of inhibition between tiaramide and other anti-inflammatory drugs.

The importance of arachidonic acid (AA) in platelet function has been established. By stimulation of collagen and thrombin, AA is hydrolyzed by phospholipase A2 (PLA2) from platelet phospholipids at the 2-acyl position. Free AA is transformed by cyclooxygenase in the microsomal fraction to the prostaglandin (PG) endoperoxide intermediates G2 and H2 which produce platelet aggregation (3). PGG2 and PGH2 are further metabolized mainly into thromboxane (TX) A2 by TX synthetase (4) in platelets.

TXA2 is a potent inducer, but is an unstable compound. In fact, potent inhibitors of platelet aggregation have been reported to depress PLA2 (mepacrine), cyclo-oxygenase (aspirin and indomethacin) and TX synthetase (OKY-1581) (5–7). Therefore, it is worthwhile to study the effect of tiaramide on the metabolic pathways of platelet phospholipids, including the breakdown into PGs and TXA2. In the experiments reported here, the mechanism by which tiaramide inhibited the aggregation was investigated using phospholipid labelling techniques by 14C-AA in rabbit platelets (8). Since tiaramide inhibited PLA2 activity in these experiments, we have additionally examined whether tiaramide affects Ca-uptake by platelets from the external medium and intracellular calcium mobilization. We
also studied whether the inhibition of PLA₂ by tiaramide is mediated through calmodulin (9, 10) using the calmodulin-dependent phosphodiesterase of rabbit brain.

**Materials and Methods**

Rabbits of either sex weighing 2.5–3.5 kg were anesthetized intramuscularly with sodium pentobarbital (30 mg/kg). Blood was taken through the carotid artery with one tenth volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by low centrifugation of the whole blood at 140 g for 12 min at room temperature.

**Measure of platelet aggregation:** Platelet aggregation was measured by continuous recording of light transmission through PRP or a platelet suspension (11). Platelet count was adjusted to 5x10⁵/M. The platelet sample was preincubated at 37°C for 3 min in an aggregometer with an inhibitor or the corresponding vehicle. AA and A23187 were dissolved in dimethylsulfoxide, and the final concentration did not exceed 0.5%.

**Assay for cyclo-oxygenase and thromboxane synthetase activity:** One ml of PRP (5x10⁵ platelets/μl) was incubated with tiaramide, aspirin, OKY-1581 or 0.9% NaCl at 37°C for 5 min. After addition of EDTA (77 mM)-saline (0.05 ml) to the PRP, the mixture was centrifuged at 2000 g for 10 min. The sediment was suspended in 0.1 M phosphate-buffered saline, pH 7.4, and incubated with ¹⁴C-AA (0.04 μCi/ml, specific activity: 58.4 mCi/mmmole), for 5 min. The sample was acidified (pH 3 to 4) with 1 N formic acid. After addition of the carriers (AA, PGD₂, PGE₂, PGF₂α and TXB₂) to the sample, platelet lipids were extracted twice with 1 ml mixture of chloroform/methanol (2:1). The platelet extract was evaporated under CO₂ gas and dissolved in chloroform (0.05 ml). An aliquot of the extract was spotted on a thin-layer chromatography (TLC) plate (Silica gel G-50) in the upper phase from a mixture of ethylacetate/isooctane/acetic acid/water (90:50:20:100, v/v, solvent system 1). Arachidonate metabolites and phospholipids (the origin) on the chromatogram were visualized by brief exposure to iodine. The radioactive zones on the plate were detected with an Aloka radiochromatogram scanner, Model JTC-203.

**Assay for phospholipase A₂ activity:** An aliquot of PRP (10⁶ platelets/μl) was incubated with ¹⁴C-AA (0.04 μCi/ml) for 90 min at 37°C. The loaded platelets were washed twice, first with Tyrode solution (pH 6.5) containing 0.2 mM ECTA and 0.35% albumin, but not CaCl₂, and second with the same solution lacking ECTA. The platelets were finally suspended in normal Tyrode solution to give a final concentration of 5x10⁵ platelets/μl. The aliquot of the labelled platelet suspension was exposed to tiaramide, mepacrine or 0.9% NaCl for 5 min, followed by collagen (30 μg/ml). Three min after collagen, platelet lipids were extracted as already described. Two kinds of solvent systems were used: One was solvent I (described above), and the other was solvent system II for separation of phospholipids which consisted of chloroform/methanol/acetic acid/water (50:30:8:4). After visualization of the substances on the plate by exposure to iodine vapour, the radioactive zones were detected with a radiochromatogram scanner and by scraping off the chromatogram in 0.5 to 1.0 cm zones. The radioactivity in each zone was estimated by liquid scintillation (Aloka liquid scintillation counter, LSC-903SP). In another experiment, 3 ml aliquots of washed platelet suspension (10⁶/μl) were incubated with 0.5 μCi of 2-(3H-arachidonyl)-phosphatidylycerine [2-(3H-arachidonyl)-PC] (specific activity; 32 Ci/mmmole). One of the samples served as a non-aggregated control; a second served as an aggregated control (30 μg collagen/ml); and each of the other 3 samples was incubated with tiaramide, mepacrine and indomethacin, respectively, for 5 min, followed by collagen. The samples were extracted, and the radioactivity was measured as described above.

**Assay for ⁴⁵Ca-uptake by rabbit platelets:** PRP (5x10⁵ platelets/μl) collected with 3.8% sodium citrate was incubated with ⁴⁵Ca (2 μCi/ml, specific activity: 0.75 mCi/mmol) for 30 min at room temperature. After 3 min preincubation at 37°C, the PRP was incubated with tiaramide, verapamil and 0.9% NaCl for 5 min. At various intervals, a 0.1 ml aliquot
of the PRP was centrifuged immediately with silicone oil SH200B/SH550 (1:7) at 9000 g for 60 sec in a Tomy microcentrifuge (MC-15A). Maximum speed was reached within 10 sec, thereby separating the platelets from the plasma and pelleting them beneath the silicone oil layer. For determination of $^{45}$Ca, the tip of a 0.4 ml polyethylene tube containing the platelet pellet was cut off and placed in a scintillation vial with 0.5 ml of 1% of Triton X-100. After overnight solubilization of the pellet at room temperature, 5 ml of scintillator was added, and the radioactivity was measured on a liquid scintillation spectrometer.

There was no significant difference between the sample in the amount of $^3$H-inulin retained by the various platelet pellets.

**Estimation of 5-hydroxytryptamine (5-HT):** Five-HT released from the platelets was estimated by the OPT method (12). The release reaction was stopped by cooling the sample in an ice water after the aggregation experiment. The sample was centrifuged at 2000 g for 10 min. The 5-HT release from the platelets was expressed as:

$$\text{% release} = \left[ \frac{(A-B)}{(C-B)} \right] \times 100$$

where, A=amount of 5-HT in the supernatant of a treated sample, B=amount of 5-HT in the supernatant of the control sample (solvent of test drug was added to the platelet suspension), and C=total amount of 5-HT in the control sample.

**Preparation of crude phosphodiesterase of rabbit brain:** Rabbit brain was homogenized with 3 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and centrifuged at 12,000 g for 10 min. The supernatant was recentrifuged at 105,000 g for 60 min. The supernatant fraction obtained was used as a source of enzyme activity. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4, 3 mM MgCl$_2$, 1.8 mM CaCl$_2$, and 2 $\mu$M cyclic AMP, in a final volume of 0.4. After 3 min preincubation at 37°C, the reaction was initiated by addition of the enzyme (50 $\mu$g protein). The reaction was continued for 5 min and terminated by addition of 0.1 N HCl and immersing the tube in boiling water for 1 min. The cyclic AMP generation was measured using a convenient radioimmunoassay kit according to the method of Honma et al. (13).

**Chemicals:** 1-$^1$C-arachidonic acid (New England Nuclear), 2-(5, 6, 8, 9, 11, 12, 14, 15-$^3$H) arachidonyl phosphatidylycerol [2-(3H-arachidonyl)-PC] (Amersham), $^3$H-(G)-inulin (New England Nuclear), $^{45}$CaCl$_2$ (Amersham-Japan), arachidonic acid (Sigma), tiaramide hydrochloride (Fujiwara), verapamil hydrochloride (Eizai), dibucaine hydrochloride (Sigma), bovine serum albumin (Sigma), 5-hydroxytryptamine creatinine sulphate (5-HT, Daiichi chemicals), O-phthalaldehyde (OPT, Wako Pure chemicals), PGF$_2$, PGF$_{2\alpha}$, PGD$_2$, TXB$_2$ and OKY-1581 (gifts by Ono Pharmaceutical Co.), phosphatidylycerol (PC) (from egg, Saradog Research Laboratories Inc., Canada), phosphatidylethanolamine (PE) (from egg yolk, Alpha Therapeutic Corporation, U.S.A.), phosphatidylserine (PS) (from bovine brain, Funakoshi Chemicals), phosphatidylinositol (PI) (from yeast, Funakoshi Chemicals), chlorpromazine hydrochloride (Shionogi), trifluoperazine maleate (Yositomi), A23187 (Eli Lilly, U.S.A.), collagen (Hormon Chemie, München), cyclic AMP (Sigma), and Silica gel G-50 (Wako Pure Chemicals).

The statistical differences between obtained values were determined using Student's $t$-test.

**Results**

**Effect of tiaramide on cyclo-oxygenase and thromboxane synthetase activity:** Since our previous study showed that tiaramide inhibited ADP- and collagen-induced aggregation (2), we studied the mechanism by which tiaramide inhibited aggregation, particularly that induced by collagen, by determining the effect of tiaramide on the cyclo-oxygenase and thromboxane synthetase activity of rabbit platelets.

Figure 1 shows TXB$_2$ (peak II) in the TLC in the tiaramide-treated platelets as well as the control platelets. In the aspirin-treated platelets, however, TXB$_2$ was not present, since platelet cyclo-oxygenase was inhibited. OKY-1581 inhibited platelet generation of TXB$_2$, but conversely increased PGD$_2$ and PGE$_2$ synthesis. Tiaramide did not decrease the amount of TXB$_2$, nor increase PGD$_2$ or
PGE2. The facts suggest that it does not affect either enzyme. In the TLC with solvent system I, the Rf value for TXB2 (peak II) was very similar to those of PGE2 (peak V) and PGD2 (peak VI) as shown in Fig. 1. We used another solvent system, ethylacetate/acetic acid (99:1), to confirm that the spot for TXB2 was different from those for PGD2 and PGE2. In this system, the Rf value for TXB2 was 0.28, and those for PGE2 and PGD2 were 0.36 and 0.18, respectively.

Effect of tiaramide on phospholipid arachidonate metabolism: Figure 2 shows the results of a series of experiments in which platelet phospholipids and AA metabolites were measured before and after aggregation by collagen and after treatment with tiaramide or mepacrine followed by collagen. As shown in Fig. 2, the radioactivity of total phospholipids (at the origin in TLC) was 85.2±3.9% of the radioactivity recovered; less than 9% was recovered as free arachidonates before aggregation in the TLC with solvent system I. After aggregation by collagen, the radioactivity of total phospholipid decreased significantly to 49.0±12.6% (P<0.05) and that of arachidonates increased (40%). Inhibition of platelet aggregation by...
tiramide (100 nM) restored total phospholipid and arachidonate levels to those before aggregation, as seen in the inhibition by mepacrine, a potent PLA₂ inhibitor (Fig. 2).

With solvent system II, 50% of the total radioactivity recovered was found in the zone of PC before aggregation; and after collagen, 35% was found in PC. The fact suggests that the liberated arachidonates originate partly in PC in the present study. In fact, the radioactivity originating in free arachidonate metabolites increased after aggregation by collagen. During the course of the experiment, we experienced difficulty in separating various phospholipids, particularly PI and PS. We measured mixed radioactivity originating in PI and PS in the extract. Figure 3 shows whole platelet PLA₂ activity stimulated by collagen (30 μg/ml) using 2-(³H-arachidonyl) PC as a substrate. In two experiments, collagen caused significant aggregation, and tiaramide, mepacrine or indomethacin inhibited the aggregation. Tiaramide decreased the amount of AA liberated from labelled PC at position 2, like mepacrine. In one experiment, indomethacin accumulated AA which was esterified collagen-stimulated PLA₂ (22% for indomethacin, 8% for the non-aggregated control).

Effect of tiaramide on collagen- and AA-induced aggregation: Figure 4 shows a typical experiment in which tiaramide (10 nM) inhibited collagen-induced aggregation, but not the AA-induced one. The concentration of tiaramide is critical for this selective blockade of collagen-induced aggregation, since higher doses of it also blocked AA-induced aggregation. Indomethacin (10 nM) blocked both AA- and collagen-induced aggregation (data not shown).

Effect of tiaramide on ⁴⁵calcium-uptake by rabbit platelets: By use of aggregation assays and the platelet phospholipid labelling technique, it is suggested that tiaramide has an inhibitory potency on the PLA₂ of rabbit platelets. Rittenhouse-Simmons et al. (14) reported that platelet PLA₂ showed maximum activity in the presence of calcium. Therefore, the effect of tiaramide was examined on calcium transference through the platelet plasma membrane. As seen in Fig. 5, tiaramide affected neither the calcium transport of the platelets nor inhibit collagen-induced calcium influx by rabbit platelets.

Additionally, the effect of tiaramide was examined on A23187-induced aggregation and 5-HT release in the presence of 1 mM EDTA to determine whether tiaramide inhibits intracellular calcium mobilization induced by A23187 (15). Tiaramide is not a potent inhibitor of A23187-induced aggregation.

Fig. 3. Radiochromatograms of 2-(³H-arachidonyl) PC metabolites. A, aggregated control (collagen 30 μg/ml); B, treatment with tiaramide (100 nM) followed by collagen; C, non-aggregated control. Abscissa, number of the 5 mm section from the origin to the front. Ordinate, % of radioactivity recovered in the 5 mm section (total radioactivity on plate was taken as 100%). Spots of non-labelled AA is shown.
and A23187-induced 5-HT release like verapamil and dibucaine (16, 17).

**Effect of tiaramide on calmodulin:** It appears that the effect of tiaramide on calmodulin needs to be examined, since Wong and Cheung (9) report that human platelet phospholipase A2 is calmodulin-dependent. Therefore, the effect of tiaramide was examined on the calmodulin-dependent phosphodiesterase activity of rabbit brain and was compared with those of chlorpromazine and trifluoperazine, well-known antagonists of calmodulin. As shown in Fig. 6, there was no effect in the presence of tiaramide; however, about 50% inhibition was seen in the presence of 30 nM chlorpromazine and trifluoperazine. The inhibitory effect of tiaramide on platelet PLA2 is not likely to be mediated by its inhibition of calmodulin.

**Discussion**

Tiaramide, a non-steroidal anti-inflammatory drug, is a potent inhibitor of platelet aggregation and of bronchoconstriction produced by histamine, bradykinin and PGF2α (18).

It has been established that platelet activation by various agents such as thrombin and collagen lead to liberation of AA from platelet phospholipids. As a result, prostaglandin-thromboxane (PG-TX) systems are activated. Various substances including PGG2, PGH2 and TXA2 in the PG-TX systems cause marked platelet aggregation. Tiaramide seems not to be an aspirin-like drug, since it did not abolish the TXE2 peak in TLC in the present study, nor reduce MDA (malondialdehyde) production (2) from prostaglandin endoperoxides during platelet aggregation. It is not a TXA2 synthetase inhibitor.
like OKY-1581, because there was no PGD2 and PGE2 generation.

Endogenous AA formation is regulated by PLA2 and this seems to be the rate-limiting step for the production of PG-TX systems in the platelets (19). The present study suggests that tiaramide has an inhibitory potency for PLA2 in rabbit platelets: in the first place, collagen-induced AA release from platelet phospholipids was effectively inhibited by tiaramide like mepacrine, and tiaramide decreased 3H-AA levels liberated from the 2 position of 2-(3H-arachidonyl)-PC; in the second place, tiaramide, while easily blocking collagen aggregation, had no effect on aggregation induced by AA; and in the third place, tiaramide had no effect on platelet cyclo-oxygenase or thromboxane synthetase.

In activated platelets, arachidonic acid is not only released from PC and PE by action of PLA2, but also released from PI by phospholipase C. AA derived from PI is released via phosphatidic acid and via diacylglyceride
As shown in Fig. 2, radioactivity originating from $^{14}$C-AA produced from degradation of labelled phospholipids is more than that originating from $^{3}$H-AA liberated from 2-(3H-arachidonyl) PC (Fig. 3). The fact suggests that the AA is liberated by cleavage of various phospholipids at position 2 containing PI. Therefore, the possibility cannot be excluded that tiaramide inhibits AA production derived from PI via its metabolites.

There are several substances which regulate PLA$_2$ activity. Cyclic-AMP and calcium ions are known as regulators. Accumulation of cyclic-AMP in the platelets is reported to inhibit PLA$_2$ activity (22, 23). The possibility that PLA$_2$ inhibition by tiaramide is mediated through cyclic-AMP accumulation must be excluded, because tiaramide decreases platelet cyclic AMP level (2).

PLA$_2$ activity in the platelet plasma membrane depends on calcium. Collagen-induced $^{45}$Ca uptake by the platelets continued throughout inhibition of the aggregation by tiaramide. Accordingly, tiaramide-induced inhibition of PLA$_2$ is not concerned with Ca-uptake by platelets.

Tiaramide inhibition of platelet aggregation, as well as the increase in $^{45}$Ca-uptake by collagen, leads us to consider that tiaramide may block influxed Ca mobilization to active sites within the platelets. Cationophores A23187 and X-537A induced platelet aggregation and the release reaction by mobilization of Ca from the intracellular depots to active sites like PLA$_2$ (15). Compared with the effects of verapamil and dibucaine, tiaramide was not a potent inhibitor except at very high concentration (16, 17). Therefore, the weak potency of tiaramide could not be the main cause for the depression of PLA$_2$ activity.

Recent evidence indicates that calmodulin is a multifunctional Ca$^{2+}$-binding protein ubiquitous in eukaryotes. Calmodulin mediates the effect of Ca$^{2+}$ on a variety of enzymes in many cellular reactions, including PLA$_2$. Wong and Cheung (9) reported that the stimulation of human platelet PLA$_2$ by Ca is indeed mediated through calmodulin. If tiaramide was bound to the complex of calmodulin and calcium like trifluoperazine and chlorpromazine, it would be reasonable that platelet PLA$_2$ is inhibited. Tiaramide, however, did not inhibit the calmodulin-dependent activation of the cyclic-AMP phosphodiesterase of rabbit brain, unlike trifluoperazine and chlorpromazine.

The mechanism by which tiaramide inhibits collagen-induced aggregation is well explained by its inhibition of PLA$_2$. This mechanism does not explain the inhibitory effect on ADP-induced aggregation. In rabbit platelets, ADP produced the first phase of aggregation without the release reaction. Therefore, the activation of the PG-TX systems does not occur during the ag-

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Table 1. Effect of tiaramide, verapamil and dibucaine on A23187-induced aggregation and 5-HT release from rabbit platelets in the presence of 1 mM EDTA

| Drugs (μM) | Platelet aggregation (%) | 5-HT release % |
|-----------|--------------------------|----------------|
| 0.9% NaCl | 64.9±2.20                | 64.6±4.50      |
| Tiaramide | 50                       | 55.2±5.56      |
|           | 100                      | 55.9±5.50      |
|           | 250                      | 44.7±8.91*     |
| Verapamil | 50                       | 63.6±1.47      |
|           | 100                      | 47.9±6.16*     |
|           | 250                      | 32.8±7.47***   |
| Dibucaine | 50                       | 54.1±5.45      |
|           | 100                      | 46.4±7.06*     |
|           | 250                      | 30.1±4.40***   |

*P<0.05, **P<0.01, ***P<0.001: Significantly different from the control.
Inhibition producing agents for ADP-induced aggregation are the PGs (PGE₁, PG₁₂ and PGD₂) (24), verapamil (25), local anesthetics (dibucaine and tetracaine) (26) and propranolol (27). These drugs inhibited the aggregation indirectly by accumulation of cyclic AMP or directly by blocking intracellular Ca mobilization. However, tiaramide did not accumulate cyclic AMP, and it did not strongly block the Ca mobilization like verapamil and dibucaine. More evidence must be accumulated to elucidate the mechanism by which tiaramide inhibits ADP-induced aggregation.

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