Biochemical Pharmacology, Vol. 41, No. 3, pp. 419–423, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

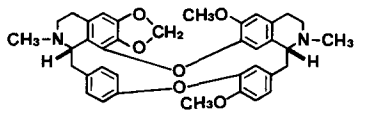
SUPPRESSIVE EFFECT OF BISCOCLAURINE ALKALOIDS ON AGONIST-INDUCED ACTIVATION OF PHOSPHOLIPASE A_2 IN RABBIT PLATELETS

TSUTOMU HASHIZUME, HIROYOSHI YAMAGUCHI, TAKASHI SATO* and TATSUZO FUJII
Department of Biochemistry, Kyoto Pharmaceutical University, Kyoto 607, Japan

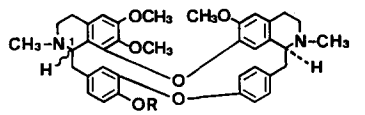
(*Received* 10 *July* 1990; *accepted* 27 *September* 1990)

Abstract—The effect of biscoclaurine (bisbenzylisoquinoline) alkaloids on phospholipase A_2 and C activation in signal transduction system of rabbit platelet was studied. Isotetrandrine, cepharanthine and berbamine inhibited the aggregation induced by collagen but not by other stimuli such as thrombin and arachidonic acid, while tetrandrine equally inhibited the aggregation by any of these agonists. All these four alkaloids suppressed arachidonic acid liberation in response to collagen or thrombin, but not diacylglycerol formation and increase in cytoplasmic Ca^{2+} concentration in response to thrombin or arachidonic acid. In saponin-permeabilized platelets, they also suppressed arachidonic acid liberation induced by an addition of both GTPγS and Ca^{2+}, whereas the liberation induced by an addition of Ca^{2+} alone was not prevented by them. These data suggest that isotetrandrine, cepharanthine and berbamine have a rather specific potency to suppress the phospholipase A_2 activation by a mechanism other than direct inhibition of the enzyme or interference with the ligand–receptor interaction. They seem, at least in part, to exert the effect on the GTP-binding protein–phospholipase A_2 complex in the platelet signal transduction system. In contrast, tetrandrine appears to inhibit a step following an increase in cytosolic free Ca^{2+} concentration in the agonist-induced signal transduction system, in addition to suppressing the phospholipase A_2 activation.

Some of the biscoclaurine (bisbenzylisoquinoline) alkaloids are shown to exert their biological activities through their membrane modifying action. These alkaloids, including cepharanthine, tetrandrine, isotetrandrine and berbamine (Fig. 1), exert inhibitory effects on platelet activation [1–4], on histamine release from rat mast cells [5], on lipid peroxidation in liposome [6] and in biological membranes [7], and on superoxide generation in polymorphonuclear leukocyte [8]. They also have transforming effect on red blood cell shape [9]. In addition to these biological effects, cepharanthine is reported to have *in vitro* antisickling effect on sickled erythrocyte [10] and to overcome resistance of certain multidrug-resistant KB carcinoma cells against anticancer agents [11].



Cepharanthine



1-S R = CH_3 Tetrandrine
1-R R = CH_3 Isotetrandrine
1-R R = H Berbamine

Fig. 1. Structure of the biscoclaurine alkaloids tested.

Our previous work has shown that one of these alkaloids, cepharanthine, suppresses collagen-induced arachidonic acid liberation as well as the aggregation [3]. We have also reported that using some synthetic benzylisoquinoline derivatives of coclaurine type, their activities to inhibit collagen-induced platelet aggregation and arachidonic acid liberation are dependent on their perturbing effect on platelet membranes [12]. In collagen stimulation, arachidonic acid liberation is suggested to be caused mainly by hydrolytic action of phospholipase A_2 on membrane phospholipids [13–15]. Our results, therefore, provide the possibility that cepharanthine and its derivatives inhibit platelet aggregation through suppression of phospholipase A_2 activation. Since other alkaloids, tetrandrine, isotetrandrine and berbamine, are also known to inhibit collagen-induced platelet aggregation [1], we examined in this work the inhibitory potencies of these four alkaloids on phospholipase A_2 activation, and further investigated their inhibitory mechanism with respect to involvement of GTP-binding protein corresponding to the enzyme.

MATERIALS AND METHODS

Washed rabbit platelets. Platelet-rich plasma was obtained from rabbit blood anticoagulated with one-tenth volume of 1% EDTA by centrifugation at 230 *g* for 10 min. The platelet-rich plasma was then centrifuged at 800 *g* for 15 min, and the platelet pellets obtained were washed twice with Ca^{2+}-free

* To whom correspondence should be addressed: Department of Biochemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan.

Tyrode–*N* - 2 - hydroxyethylpiperazine - *N'* - 2-ethanesulfonic acid (HEPES) buffer (137 mM NaCl, 2.7 mM KCl, 2.9 mM NaH_2PO_4, 1 mM $MgCl_2$, 3.8 mM HEPES, 5.6 mM glucose, 0.4 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), pH 6.5), containing 0.35% bovine serum albumin (BSA). Finally, the cell suspension was adjusted to 5×10^8 cells/mL in the same buffer without EGTA (pH 7.4).

Platelet aggregation. Platelet aggregation was continuously monitored as a change in light transmission in an aggregometer (NKK Hema Tracer 1, Niko Bioscience Co., Japan).

Lipid analysis in agonist-stimulated platelets. The platelet-rich plasma was incubated with [^{3}H]arachidonic acid (2 μCi/mL) at 37° for 1 hr and washed as described above to obtain [^{3}H]arachidonic acid-labelled platelets. The platelets pretreated with each alkaloid at 37° for 2 min in the presence of 50 μM BW755C and 1 mM $CaCl_2$, were exposed to collagen (50 μg/mL) or thrombin (0.5 units/mL) for an appropriate time. The reactions were terminated by addition of chloroform/methanol/HCl (200:200:1, by vol.), and then the lipids were extracted and analysed by TLC on Silica Gel G plates with a developing solvent, petroleum ether/diethyl ether/acetic acid (60:45:1, by vol.). Each fraction corresponding to free fatty acid and diacylglycerol was identified by comigration with each authentic standard. The areas were scraped off and the radioactivity was determined by liquid scintillation counting.

Phospholipase A_2 activity in saponin-permeabilized platelets. [^{3}H]Arachidonic acid-labelled platelets were adjusted to 2.5×10^9 cells/mL in Tyrode–HEPES buffer without BSA and EGTA (pH 7.2). Just before use, the labelled platelet suspension was diluted 5-fold with buffer (160 mM KCl, 2.3 mM $MgCl_2$, 12 mM HEPES, pH 7.2). The platelets, pretreated with 50 μM BW755C at 37° for 2 min, were incubated with each alkaloid or *p*-bromophenacyl bromide for 2 min, and then with saponin (5–6.5 μg/mL) for an additional 2 min. The permeabilized platelets were exposed to 500 μM $CaCl_2$ and 100 μM GTPγS, or various concentrations of $CaCl_2$ without GTPγS at 37° for 15 min, and [^{3}H]arachidonic acid released was measured as described above.

Reagents. Collagen (type 1) and arachidonic acid were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Thrombin (bovine plasma) was from Mochida Pharmaceutical Co., Ltd (Japan). Bovine serum albumin (BSA, fraction V) was from Boehringer Mannheim GmbH (Mannheim, F.R.G.). *p*-Bromophenacyl bromide was from Wako Pure Chemical Industries Ltd. (Japan). [^{3}H]Arachidonic acid (95.1 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Cepharanthine, tetrandrine, isotetrandrine and berbamine were given by Kaken Syoyaku Co., Ltd (Japan). Other reagents were obtained from commercial sources.

RESULTS

Effect of the alkaloids on platelet aggregation

Effect of the alkaloids tested on platelet aggregation induced by collagen, arachidonic acid or thrombin is shown in Fig. 2. Because collagen- or arachidonic acid-induced aggregation was blocked by aspirin or indomethacin under the condition used here (data not shown), the responses induced by these agonists were recognized to be dependent of thromboxane A_2 generation. All the alkaloids inhibited dose-dependently the collagen-induced aggregation. However, the concentrations required to inhibit the aggregation almost completely were 20 μM for isotetrandrine and tetrandrine and 40 μM for cepharanthine and berbamine (Fig. 2A). On the other hand, these alkaloids except tetrandrine had little effect on arachidonic acid or thrombin stimulation even at 40 μM (Fig. 2B and C), indicating their specific inhibitory potency for collagen stimulation (up to 40 μM tested). In contrast, tetrandrine strongly inhibited the aggregation by any of these agonists, that is, it appears to have no specificity for each agonist.

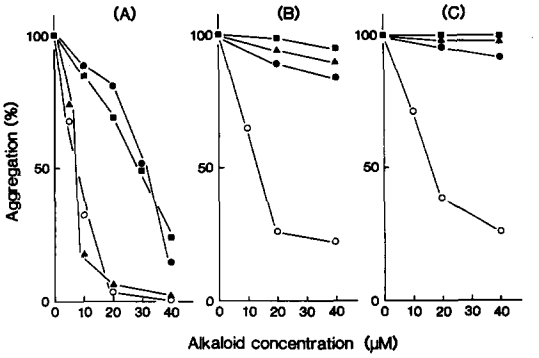


Fig. 2. Inhibition by each alkaloid of platelet aggregation induced by 5 μg/mL collagen (A), 100 μM arachidonic acid (B) or 0.1 units/mL thrombin (C). Washed platelets were incubated with various concentrations of cepharanthine (●), tetrandrine (○), isotetrandrine (▲) or berbamine (■) at 37° for 2 min, and then stimulated with each agonist. The light transmission of the platelets after 5 min stimulation in the absence of the alkaloid was taken as 100%. Each point represents the average of two separate experiments performed in duplicate.

Effect of the alkaloids on phospholipase A_2 and C activation

The agonist-induced increase in phospholipase activities plays an important role in platelet activation induced by collagen or thrombin. Therefore, to estimate the inhibitory mechanism of the alkaloids tested on agonist-induced platelet activation, their effect on phospholipase A_2 and C activation in response to collagen or thrombin was studied. As shown in Fig. 3, when [^{3}H]arachidonic acid-labelled platelets were pretreated with each alkaloid and exposed to collagen or thrombin, [^{3}H]arachidonic acid liberation was inhibited dose-dependently. The potency of tetrandrine and isotetrandrine to inhibit the collagen-induced liberation seems to be higher than that of cepharanthine and berbamine (Fig. 3A). This result is consistent with that obtained in the experiment of collagen-induced aggregation. Interestingly, all the alkaloids inhibited thrombin-induced

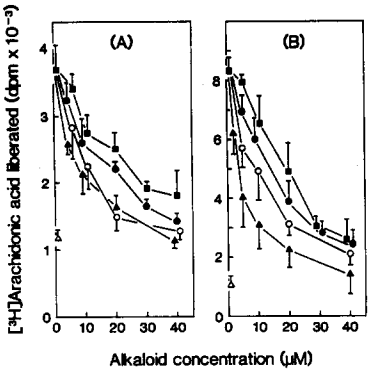


Fig. 3. Inhibition by each alkaloid of arachidonic acid liberation induced by collagen (A) or thrombin (B). [^{3}H]Arachidonic acid-labelled platelets were incubated with various concentrations of cepharanthine (●), tetrandrine (○), isotetrandrine (▲) or berbamine (■) at 37° for 2 min in the presence of 50 μM BW755C, and then stimulated with 50 μg/mL collagen for 1 min or 0.5 units/mL thrombin for 30 sec. [^{3}H]Arachidonic acid liberated was determined as described in Materials and Methods. Each point represents the mean ± SD of three separate experiments performed in duplicate: △; without stimulation.

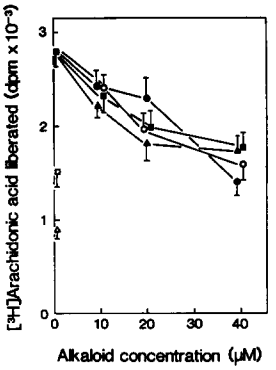


Fig. 5. Inhibition of GTPγS-induced arachidonic acid liberation by each alkaloid in saponin-permeabilized platelets. [^{3}H]Arachidonic acid-labelled platelets were incubated with various concentrations of cepharanthine (●), tetrandrine (○), isotetrandrine (▲) or berbamine (■) at 37° for 2 min in the presence of 50 μM BW755C, and then treated with saponin. After 2 min, 100 μM GTPγS and 500 μM $CaCl_2$ were added and incubated for 15 min, and [^{3}H]arachidonic acid liberated was determined as described in Materials and Methods. Each point represents the mean ± SD of three separate experiments performed in duplicate. □; without GTPγS, △; without both GTPγS and $CaCl_2$.

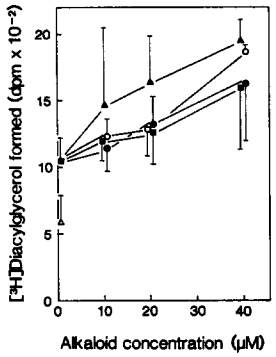


Fig. 4. Effect of each alkaloid on diacylglycerol formation induced by thrombin. The experimental conditions and symbols are the same as those in Fig. 3. [^{3}H]Diacylglycerol formed was determined as described in Materials and Methods. Each point represents the mean ± SD of three separate experiments performed in duplicate.

liberation at the similar concentration range as in collagen stimulation (Fig. 3), though alkaloids other than tetrandrine did not depress thrombin-induced aggregation (Fig. 2C). Since arachidonic acid liberation in response to collagen or thrombin is reported to occur mainly through the action of phospholipase A_2 on membrane phospholipids [13–17], the results obtained here indicate the suppression of the enzyme activation by each alkaloid.

On the other hand, no suppressive effect of these alkaloids was observed on thrombin-induced diacylglycerol formation (Fig. 4). In addition, tetrandrine did not prevent the increase in cytosolic free Ca^{2+} concentration in the quin 2-loaded platelets in response to thrombin and arachidonic acid even at the concentration of 40 μM, that almost completely inhibited platelet aggregation in response to such agonist (data not shown).

Effect of the alkaloids on GTPγS-induced phospholipase A_2 activation

We and some other investigators recently suggested that phospholipase A_2 activation induced by collagen or thrombin might be modulated by each putative GTP-binding protein [18–22]. Therefore, the possibility that the alkaloids inhibit phospholipase A_2 through interference with GTP-binding protein–enzyme association, was examined. As shown in Fig. 5, GTPγS-induced [^{3}H]arachidonic acid liberation in [^{3}H]arachidonic acid-labelled, saponin-permeabilized platelets, was suppressed dose-dependently by each alkaloid at the concentration up to 40 μM. However, the maximum inhibition at 40 μM of each seems to remain at the level of the liberation that is induced by an addition of Ca^{2+} alone without GTPγS. This suggests that the alkaloids may not affect the enzyme molecule or enzyme-substrate interaction directly. Hence, we further studied the effect of alkaloids on Ca^{2+}-induced phospholipase A_2 activation in comparison with *p*-bromophenacyl bromide, known to inhibit the enzyme activity directly [23]. The results of Fig. 6 demonstrate that *p*-bromophenacyl bromide prevented Ca^{2+}-induced arachidonic acid liberation, while all the alkaloids tested failed to do the same.

DISCUSSION

In the sequence of stimulus–response coupling in

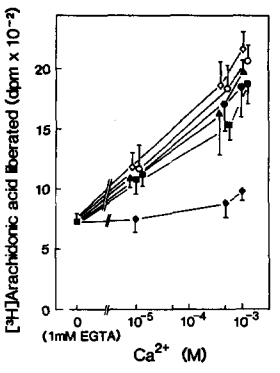


Fig. 6. Comparison of the effect of each alkaloid on Ca^{2+}-activated phospholipase A_2 activity with that of *p*-bromophenacyl bromide. [^{3}H]Arachidonic acid-labelled platelets were incubated with 40 μM of cepharanthine (●), tetrandrine (○), isotetrandrine (▲), berbamine (■) or *p*-bromophenacyl bromide (◆), or DMSO (vehicle, ◇) at 37° for 2 min in the presence of 50 μM BW755C, and then treated with saponin. After 2 min various concentrations of $CaCl_2$ were added in the presence of 1 mM EGTA, and [^{3}H]arachidonic acid liberated was determined as described in Materials and Methods. Free Ca^{2+} concentrations calculated were expressed on the horizontal axis. Each point represents the mean ± SD of three separate experiments performed in duplicate.

platelets, phospholipase A_2 activation is essential as the earliest step in collagen-induced aggregation, but not in arachidonic acid- or thrombin-induced aggregation. The present study revealed that at the concentration ranges tested (up to 40 μM) isotetrandrine, cepharanthine and berbamine specifically inhibited the aggregation induced by collagen but not by arachidonic acid and thrombin, whereas tetrandrine inhibited the aggregation induced by thrombin or arachidonic acid in addition to collagen. Furthermore, these alkaloids inhibited arachidonic acid liberation from membrane phospholipids in response to collagen and thrombin at the concentration ranges that inhibited collagen-induced aggregation. These data demonstrate that isotetrandrine, cepharanthine and berbamine have a specific action to inhibit platelet phospholipase A_2 activation, whereas tetrandrine appears to suppress another process as well, because the concentrations to inhibit the enzyme are almost the same as those to inhibit arachidonic acid- and thrombin-induced aggregation which can be caused even in the absence of phospholipase A_2 activation. In addition, isotetrandrine appears to be more effective for the inhibition of phospholipase A_2 than cepharantine and berbamine, since it inhibited both collagen-induced aggregation and arachidonic acid liberation at lower concentration than the latter compounds.

On the other hand, all the alkaloids tested did not influence diacylglycerol formation and increase in cytosolic free Ca^{2+} concentration induced by thrombin or arachidonic acid, which are to be induced by phospholipase *C* activation following ligand–receptor interaction. This result suggests that these alkaloids have no suppressive effects on phospholipase C activity and also ligand–receptor interaction such as thrombin and thromboxane A_2. Therefore, the lack of effect of isotetrandrine, cepharanthine and berbamine on this enzyme appears to explain their almost no effect on arachidonic acid- and thrombin-induced aggregation. Nevertheless, a strong potency of tetrandrine to inhibit aggregation induced by these agonists was observed, suggesting that it inhibited aggregation by suppressing a process following an increase in cytosolic free Ca^{2+} concentration which is a common step for every agonist used in their signal transduction system, such as an assembly of cytoskeletal proteins, as suggested in our previous studies [24, 25]. Teh *et al.* [4] recently suggested that tetrandrine inhibits aggregation by an interference with the phosphatidylinositol metabolism. In the present study, however, we could not observe such an effect of tetrandrine, because it had no effect on diacylglycerol formation and increase in cytosolic Ca^{2+} induced by thrombin.

We and some other investigators recently suggested that collagen- and thrombin-induced phospholipase A_2 activation might be modulated by each putative GTP-binding protein [18–22]. The present results indicated that GTPγS-induced arachidonic acid liberation in saponin-permeabilized platelets was inhibited by any of these alkaloids. However, they could not inhibit the liberation induced by an addition of high concentration of Ca^{2+} alone, whereas *p*-bromophenacyl bromide, a compound that is known to inhibit phospholipase A_2 directly, inhibited it significantly under the same condition. These evidences suggest that the inhibition of arachidonic acid liberation by the alkaloids tested may not be due to the direct inhibition of the enzyme. Although the precise mechanism underlying these effects remains to be elucidated, they seem to interfere with the GTP-binding protein-associated phospholipase A_2 system, probably through perturbation of lipid phase surrounding these two proteins, which might influence the certain functionally important microdomains of the proteins. No differences were observed in the inhibitory potencies of isotetrandrine, cepharanthine and berbamine on GTPγS-induced phospholipase A_2 activation, while isotetrandrine inhibited agonist-induced activation of the enzyme in a lower concentration than other two alkaloids. This observation may be interpreted to suggest that isotetrandrine affects the association of receptor-GTP-binding protein, in addition to the effect on the coupling of GTP-binding protein and phospholipase A_2 for signal transduction.

In conclusion, we demonstrated in this work that isotetrandrine, cepharanthine and berbamine have a specific potency to inhibit phospholipase A_2 activation and that the inhibition is brought by a mechanism other than direct inhibition of the enzyme molecule or of the ligand–receptor interaction, probably through suppression of signal transduction involved in the corresponding GTP-binding protein–phospholipase A_2 system. In contrast, tetrandrine seems to inhibit a process following an increase in cytosolic free Ca^{2+} concentration in the agonist-induced signal transduction system, in addition to suppressing the phospholipase A_2 activation.

REFERENCES

1. Watanabe S, Morimoto YM, Shiraishi N, Sano A and Utsumi K, The inhibition of platelet aggregation by biscoclaurine alkaloids. *Cell Struct Funct* **6**: 263–267, 1981.
2. Kanaho Y, Sato T and Fujii T, Mechanism of the inhibitory effect of cepharanthine on the aggregation of platelets. *Cell Struct Funct* **7**: 39–48, 1982.
3. Kometani M, Kanaho Y, Sato T and Fujii T, Inhibitory effect of cepharanthine on collagen-induced activation in rabbit platelets. *Eur J Pharmacol* **111**: 97–105, 1985.
4. Teh BS, Ioannoni B, Seow WK, McCormack JG and Thong YH, Suppression by tetrandrine of human platelet aggregation induced by platelet-activating factor and other stimulants. *Int Arch Allergy Appl Immunol* **88**: 267–272, 1989.
5. Teh BS, Seow WK, Chalmers AH, Playford S, Ioannoni B and Thong YH, Inhibition of histamine release from rat mast cells by the plant alkaloid tetrandrine. *Int Arch Allergy Appl Immunol* **86**: 220–224, 1988.
6. Nagatsuka S and Nakazawa T, Effects of membrane-stabilizing agents, cholesterol and cepharanthin, on radiation-induced lipid peroxidation and permeability in liposomes. *Biochim Biophys Acta* **691**: 171–177, 1982.
7. Shiraishi N, Arima T, Aono K, Inouye B, Morimoto Y and Utsumi K, Inhibition by biscoclaurine alkaloid of lipid peroxidation in biological membranes. *Physiol Chem Physics* **12**: 299–305, 1980.
8. Matsuno T, Orita K, Sato E, Nobori K, Inoue B and Utsumi K, Inhibition of metabolic response of polymorphonuclear leukocyte by biscoclaurine alkaloids. *Biochem Pharmacol* **36**: 1613–1616, 1987.
9. Sato T, Kanaho Y and Fujii T, Relation of the characteristic action of biscoclaurine alkaloids on the erythrocyte membrane and their incorporation into the membrane. *Cell Struct Funct* **5**: 155–163, 1980.
10. Sato T and Ohnishi ST, *In vitro* anti-sickling effect of cepharanthine. *Eur J Pharmacol* **83**: 91–95, 1982.
11. Shiraishi N, Akiyama S, Nakagawa M, Kobayashi M and Kuwano M, Effect of bisbenzylisoquinoline (biscoclaurine) alkaloids on multidrug resistance in KB human cancer cells. *Cancer Res* **47**: 2413–2416, 1987.
12. Fujii T, Sato T, Tamura A, Kometani M, Nakao K, Fujitani K, Kodama K and Akasu M, Structure–activity relationships of 4′-*O*-substituted 1-benzylisoquinolines with respect to their actions on the cell membrane of blood platelets and erythrocytes. *Eur J Pharmacol* **146**: 285–290, 1988.
13. Pollock WK, Rink TJ and Irvine RF, Liberation of [^{3}H]arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. *Biochem J* **235**: 869–877, 1986.
14. Vedelago HR and Mahadevappa VG, Mobilization of arachidonic acid in collagen-stimulated human platelets. *Biochem J* **256**: 981–987, 1988.
15. Sato T, Akiba S and Fujii T, Effect of phorbol 12-myristate 13-acetate on collagen-induced signal transduction in rabbit platelet. *Thromb Res* **49**: 567–579, 1988.
16. Mahadevappa VG and Holub BJ, Diacylglycerol lipase pathway is a minor source of released arachidonic acid in thrombin-stimulated human platelets. *Biochem Biophys Res Commun* **134**: 1327–1333, 1986.
17. Purdon AD, Patelunas D and Smith JB, Evidence for the release of arachidonic acid through the selective action of phospholipase A_2 in thrombin-stimulated human platelets. *Biochim Biophys Acta* **920**: 205–214, 1987.
18. Nakashima S, Hattori H, Shirato L, Takenaka A and Nozawa Y, Differential sensitivity of arachidonic acid release and 1,2-diacylglycerol formation to pertussis toxin, GDPβS and NaF in saponin-permeabilized human platelets: Possible evidence for distinct GTP-binding proteins involving phospholipase C and A_2 activation. *Biochem Biophys Res Commun* **148**: 971–978, 1987.
19. Fuse I and Tai H-H, Stimulations of arachidonate release and inositol-1,4,5-triphosphate formation are mediated by distinct G-proteins in human platelets. *Biochem Biophys Res Commun* **146**: 659–665, 1987.
20. Akiba S, Sato T and Fujii T, Differential effects of phorbol 12-myristate 13-acetate on GTPγS-induced diacylglycerol formation and arachidonic acid liberation in saponin-permeabilized rabbit platelets. *Thromb Res* **53**: 503–512, 1989.
21. Kajiyama Y, Murayama T and Nomura Y, Pertussis toxin-sensitive GTP-binding proteins may regulate phospholipase A_2 in response to thrombin in rabbit platelets. *Arch Biochem Biophys* **274**: 200–208, 1989.
22. Silk ST, Clejan S and Witkom K, Evidence of GTP-binding protein regulation of phospholipase A_2 activity in isolated human platelet membranes. *J Biol Chem* **264**: 21466–21469, 1989.
23. Roberts MF, Deems RA, Mincey TC and Dennis EA, Chemical modification of the histidine residue in phospholipase A_2 *(Naja naja naja)*. A case of half-site reactivity. *J Biol Chem* **252**: 2405–2411, 1977.
24. Kometani M, Sato T and Fujii T, Effect of membrane-interacting amphiphiles on association of membrane glycoproteins with assembled cytoskeletal proteins in concanavalin A-activated rabbit platelets. *Thromb Res* **42**: 567–577, 1986.
25. Sato T, Kometani M and Fujii T, Certain membrane-interacting amphiphiles inhibit aggregation and reverse shape change of rabbit platelets pre-activated with arachidonic acid through dissociation of cytoskeletal assembly. *Thromb Res* **46**: 587–592, 1987.