EXPERIMENTAL STUDY

Pharmacological intervention with platelet phospholipase A_2

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Abstract

Background: **Metabolites of arachidonic acid are important regulatory substances in blood platelets. They participate in platelet adhesion and aggregation, and pharmacological intervention with arachidonate cascade is widely used in therapy of hyperactive platelets and in the prevention of thromboembolic complications.**

Aim of the study: **To verify and compare the effect of cationic amphiphilic drugs (CAD) from different pharmacological groups on activation of platelet phospholipase A_2 — the essential enzyme of arachidonic pathway in blood platelets.**

Methods**: Blood platelets were isolated from human and rat blood by differential centrifugation and aggregation was measured in pretreated and subsequently stimulated platelets in a dual channel aggregometer and recorded on a linear recorder. Activity of cytosolic phospholipase A_2 ($cPLA_2$) was determined by means of arachidonic acid liberation from platelet membrane phospholipids incorporated as triciated radionuclide. The radioactivity was determined by liquid scintillation method in Packard TriCarb 2500T.**

Results**: Cationic amphiphilic drugs of the beta-adrenoceptor blocking group inhibited platelet aggregation in the rank order of potency: propranolol>alprenolol>metipranolol>atenolol. Similarly did the H_1-histamine antagonists bromadryl and dithiaden as well as the antimalarial chloroquine show antiplatelet effect in vitro in the rank order of potency: dithiaden≥bromadryl≥chloroquine. Dose-dependent inhibition of aggregation was followed by inhibition of arachidonic acid liberation from membrane phospholipids of platelets stimulated at receptor site (thrombin) or by a stimulus bypassing membrane receptors (Ca^{2+} ionophore A23187). The rank order potency for inhibition of stimulated ^{3}H-AA liberation from membrane phospholipids was: a) for BAB drugs: propranolol≥alprenolol>metipranolol, b) for other drugs: dithiaden>bromadryl>chloroquine.**

Conclusion: **The dugs investigated interference with liberation of stimulated arachidonic acid showed nonspecific inhibition of platelet cytosolic phospholipase A_2 by these drugs at intracellular level. The results revealed that besides inhibition of cyclooxygenase pathway, and of receptors for ADP and glycoproteins Gp IIb/IIIa, interaction of drugs with $cPLA_2$ may represent a further site for antiplatelet action. *(Fig. 5, Ref. 39.)***

Key words: blood platelets, ß-blockers, H_1-histamine antagonists, chloroquine, aggregation, cytosolic phospolipase A_2.

Arachidonic acid (AA) cascade represents the most important pathway for blood platelet (BP) activation. Stimulation of BP with different stimuli, such as thrombin, collagen, adenosinediphosphate or Ca^{2+}-ionophore A23187 resulted in early activation of Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$ — EC 3.1.1.4), which hydrolyses AA from membrane phospholipids of BP (11). After splitting due to $cPLA_2$, AA acts as second messenger and provides the substrate which is metabolised par-

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ticularly via cyclooxygenase pathway to form endoperoxides, prostaglandins and final products — thromboxanes (7, 21). Thromboxanes generated by BP represent one of the most effective endogenous autoaggregatory and vasoconstrictory mediators, which play an important role when increased during ischaemic heart disease and atherosclerosis (1, 10). Despite the existence of modern, highly active antiplatelet drugs of the 3rd generation (e.g. inhibitors of membrane glycoproteins Gp IIb/IIIa) (31), the pharmacological intervention at AA cascade remains attractive for clinical events accompanied with hyperactivation of BP. It has been demonstrated that drugs interfering with AA pathway represent the most effective, safe and available substances for long lasting prevention of cardio- and cerebrovascular diseases (6).

We found that many cationic amphiphilic drugs (CAD) with different pharmacological properties inhibit platelet aggregation, AA liberation, malondialdehyde production and thromboxane A_2 generation in blood platelets in vitro (15, 17, 29, 30). In this work we compared the antiaggregatory effect with inhibition of $cPLA_2$ in platelets pretreated with betaadrenoceptor blocking drugs (BAB drugs — alprenolol, atenolol, metipranolol, practolol, propranolol, atenolol), two H_1-histamine receptor antagonists (bromadryl, dithiaden), and the antimalarial chloroquine with antiinflammatory and immunomodulatory properties.

Methods and materials

Blood sampling from rats

The method described previously was applied (25). Briefly, male 350±30 g Wistar rats were used. Blood in the amount of 9 ml was taken through a polyethylene catheter from the a. carotis communis in light ether anaesthesia to polyethylene tubes with 1 ml of 0.129 mmol/l trisodium citrate. To get platelet rich plasma (PRP), blood was centrifuged 15 min at 250xg and 22 °C. Transferred PRP was centrifuged 10 min at 1000xg and 22 °C to separate platelets. Washing of platelets and the preparation of samples for aggregation followed the procedure described below, except that the final dilution of platelets was made so as to get $2.5x10^5$ platelets/µl of the sample.

Blood sampling from volunteers

Blood was taken at the blood bank from healthy volunteers (men, aged 20 to 50 years) by antecubital venepuncture and was immediately mixed with 3.8 % v/w trisodium citrate dihydrate, ratio 9 ml of blood to 1 ml of citrate in polypropylene centrifuge tubes.

Centrifugation

Blood was centrifuged 30 to 40 min after venepuncture for 15 min at 200xg and 22 °C. Platelet rich plasma was removed and the blood was re-centrifuged for 30 min at 2200xg at 22 °C to obtain platelet poor plasma (PPP). This was used to adjust the reference point in the aggregometer and to dilute the PRP. The number of platelets was adjusted after counting in a Thrombocounter Coulter.

Aggregation of isolated platelets

Whole blood with 3.8 % w/v trisodium citrate dihydrate was centrifuged for 15 min at 200xg at 22 °C. PRP was transferred and mixed with a mixture of 4.5 % w/v citric acid and 6.6 % w/v dextrose at 50 µl per 1 ml of PRP. After centrifugation at 980xg for 10 min at 22 °C, PPP was decanted and platelets were resuspended in an equal volume of PPP with Tyrode solution +EDTA (see Materials). After stabilising the suspension of platelets for 10 min at room temperature, the samples were centrifuged for 6 min at 980xg and 22 °C. Sedimented platelets were resuspended in an equal volume of Tyrode solution without EDTA and the suspension was adjusted with Tyrode solution to obtain 200 000 platelets per 1 µl. For aggregation studies 450 µl of platelet suspension/sample was stabilised for 2 min at 37 °C, the drug tested was added (20 µl) and incubated for 30 s. Aggregation was induced either with calcium ionophore A23187 (20 µl, final concentration 1.8 µmol/l), or thrombin (20 µl, final concentration 0.05 NIH U/mll) and was recorded for 2.5 min. The development and duration of aggregation was registered on linear recorder during 2.5 min. Changes in aggregation were evaluated from the amplitude of aggregation curves in (mm) at 30 s and compared with control amplitude (only stimulus = 100 %).

Phospholipase A_2 activation (measured as ^{3}H-arachidonic acid liberation)

The method recently described by Nosal et al. (32) was used. The PRP was mixed with a mixture of 4.5 w/v citric acid and 6.6 % w/v dextrose in the amount of 50 µl per 1 ml PRP and with [^{3}H]-arachidonic acid (^{3}H-AA) $1.85x10^4$ Bq/ml of PRP. After 60 min incubation at 37 °C, the samples were centrifuged for 10 min at 980xg and 22 °C and the platelets were washed two times by centrifugation in an equal volume of Tyrode buffer with EDTA for 6 min at 980xg at 22 °C. The supernatant was removed and the platelets were resuspended in Tyrode buffer without EDTA and after counting it was adjusted with Tyrode buffer to get 400 000 platelets per 1 µl of sample. The 1 ml samples were stabilised for 2 min at 37 °C, the drug tested was added (20 µl) and incubated for 30 s. The stimulation was induced with A23187 (20 µl, final concentration 1.8 µmol/l), or thrombin (20 µl, final concentration 0.05 NIH U/ml) for 5 min at 37 °C.

The incubation was terminated by centrifugation of the samples at 12 000xg for 3 min at 4 °C. After removal of the supernatant the sedimented platelets were resuspended in 0.5 ml of deionised H_2O and 2 ml of the extraction mixture, which consisted of chloroform, methanol and H_2O deion. in the ratio 20:40:1. After vigorous mixing 0.5 ml of H_2O deion. and 0.5 ml chloroform were added to each sample and the tubes were centrifuged at 2200xg for 30 min at 22 °C.

The chloroform layer was removed and 400 µl from both the supernatant and the sediment extract were transformed to 5 ml of Bray's scintillation fluid to determine the radioactivity in a Packard Tricarb 2500 scintillation counter. The amount of ^{3}H-AA liberation was evaluated as percentage from the total radioactivity in the sample, as described previously (15).

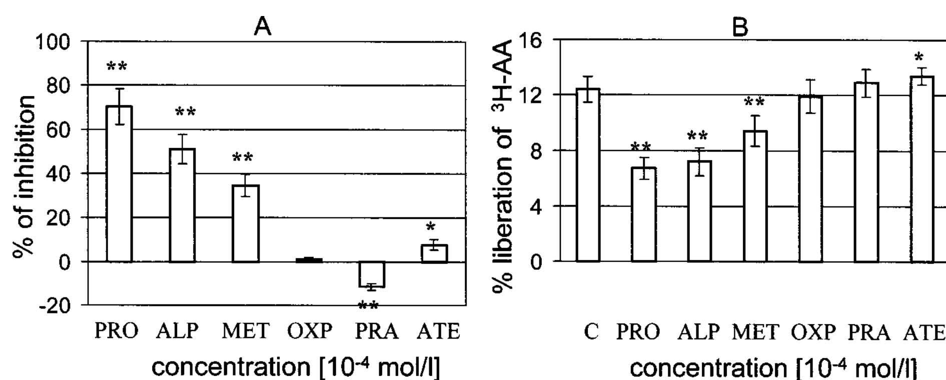

Fig. 1. Effect of ß-adrenergic receptor blocking drugs alprenolol (ALP), atenolol (ATE), metipranolol (MET), oxprenolol (OXP), practolol (PRA) and propranolol (PRO) on aggregability (panel A) and activity of cytosolic phospholipase A_2 (panel B) in isolated blood platelets stimulated with thrombin. n = 4 to 8, x±SEM, * p≤0.05; ** p≤0.01, C = control (thrombin).

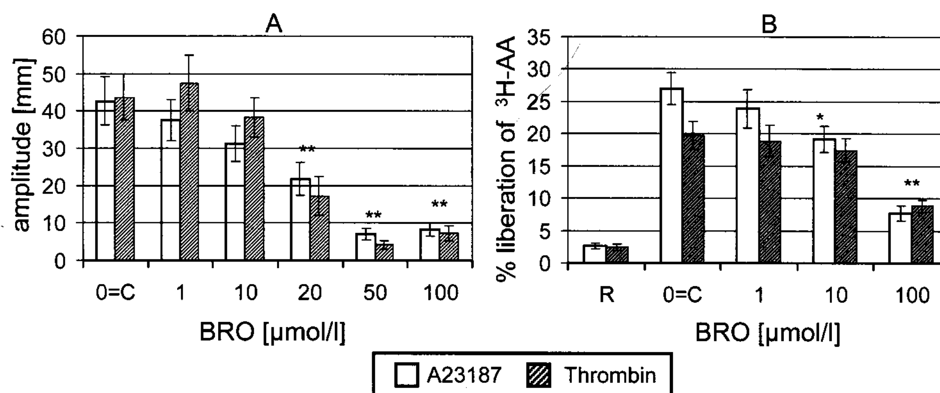

Fig. 2. Dose-dependent effect of bromadryl (BRO) on aggregation (panel A) and arachidonic acid (AA) liberation from membrane phospholipids of blood platelets (panel B) stimulated with A23187 and thrombin. n = 4 to 6, x±SEM, * p≤0.05, ** p≤0.01, R = resting platelets, C = control.

Materials

Alprenolol: AB Hässle (Hälsingborg, Sweden), atenolol: Astra-Zeneca (So-dertalje, Sweden), chloroquine: ACO (Molndal, Sweden), bromadryl, dithiaden, metipranolol: VÚFB (Praha), oxprenolol: CIBA-Geigy, (Basel Sweitzerland), practolol, propranolol: ICI Alderley Park; UK, calcium ionophore A23187: Calbiochem (La Jolla, CA, USA), human thrombín: Imuna (Šarišské Michaľany), Bray scintillation solution: Spolana (Neratovice), [3,5,8,9,11,14,15 (N)^{3}H] — arachidonic acid (7 TBq/mmol/l): Institute of Isotopes (Budapest - a kind gift from Dr. I. Mucha).

All other chemicals were from available commercial sources. Tyrode solution: 137 mmol/l NaCl, 2.7 mmol/l KCl, 12 mmol/l $NaHCO_3$, 0.4 mmol/l NaH_2PO_4x12 H_2O, 1 mmol/l $MgCl_2$x6 H_2O, 5.4 mmol/l EDTA, 5.6 mmol/l dextrose, pH 6.9.

Statistical evaluation: The data from measurements were calculated in the MS Windows '98 operating PC programmes (Excel, Statistica) and evaluated statistically by means of Student's *t*-test. The evaluation of ED_{50} values was made by means of a programme Pharm CP vers.1989.

Results

Figure 1 demonstrates the effect of BAB drugs on aggregation (panel A) and on ^{3}H-AA liberation (panel B) in isolated platelets stimulated with thrombin. In both parts of the figure drugs are ranked according to their activity. It is evident that propranolol, alprenolol, metipranolol and atenolol in the concentration of 10^{-4} mol/l significantly decreased thrombin-induced aggregation to 70, 51, 35 and 7.7 % of the control (thrombin alone), respectively. Oxprenolol did not show significant inhibition and practolol slightly potentiated the effect of thrombin on platelet aggregation.

Thrombin liberated approximately 12 % from the total AA incorporated into membrane phospholipids. Propranolol, alpre-

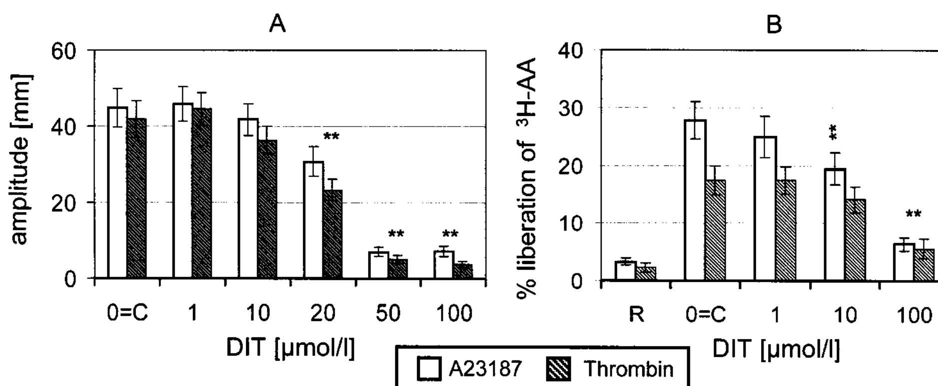

Fig. 3. Effect of dithiaden (DIT) on aggregability (expressed as the amplitude of aggregation curves, panel A) and activity of $cPLA_2$ (measured by means of arachidonic acid-AA liberation — panel B) in human blood platelets stimulated with thrombin and A23187 in vitro. n = 4 to 6±SEM, ** p≤0.01, C = control, R = resting platelets.

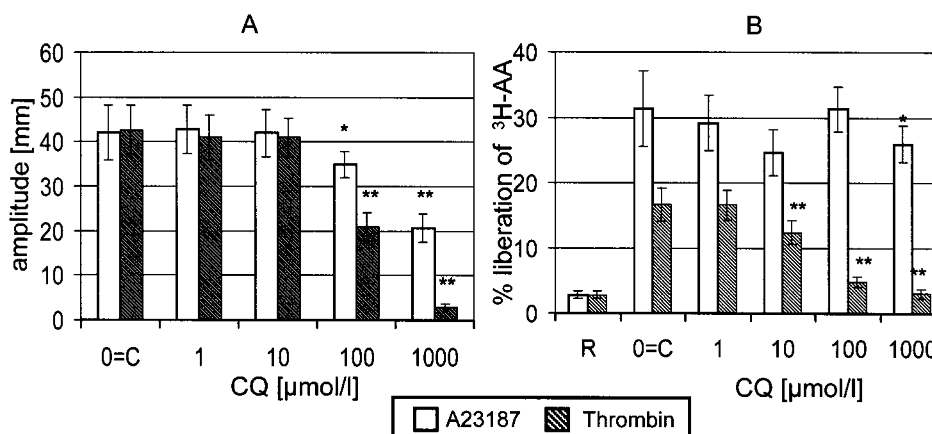

Fig. 4. Dose-dependent effect of chloroquine on aggregability (expressed as the amplitude of aggregation curves, panel A) and on activity of $cPLA_2$ (measured by means of arachidonic acid-AA liberation — panel B) from human blood platelets stimulated in vitro with thrombin and A23187. n = 4 to 6±SEM, * p≤0.05, ** p≤0.01, C = control, R = resting platelets.

nolol and metipranolol significantly decreased thrombin-induced liberation of AA to 6.7, 7.2 and 9.4 %, respectively. Oxprenolol and practolol did not affect thrombin-stimulated liberation of AA while atenolol and practolol potentiated the effect of thrombin to 13.4 %.

Effect of bromadryl (BRO) on aggregation and ^{3}H-AA liberation from isolated platelets stimulated with thrombin and A23187 is presented on Figure 2. BRO dose-dependently decreased the amplitude of aggregation curves of platelets stimulated either with thrombin or with A23187 (panel A). A significant decrease in the amplitude appeared with BRO in concentration of 20 µmol/l and this effect was potentiated on increasing the concentration of BRO to 50 and 100 µmol/l. BRO proportionally decreased the effect of both stimuli.

Pretreatment of platelets with BRO resulted in a dose-dependent decrease of ^{3}H-AA liberation induced with thrombin and A23187. A significant decrease in the AA liberation appeared with BRO in the concentration of 10 µmol/l and a further decrease with BRO in 100 µmol/l concentration is evident (Fig. 2, panel B).

A concentration-dependent effect of dithiaden (DIT) on aggregation and ^{3}H-AA liberation from thrombin- and A23187 stimulated platelets is demonstrated in Figure 3. Similarly to BRO, DIT significantly decreased the amplitude of aggregation curves after both stimuli starting at the concentration of 20 µmol/l (panel A) and the liberation of AA at 10 µmol/l concentration (panel B).

Chloroquine (CQ) (Fig. 4) significantly decreased the amplitude of aggregation curves in A23187- and trhombin-stimulated platelets at the concentration of 100 µmol/l. This effect was pronounced with 1000 µmol/l CQ in platelets stimulated with thrombin (panel A). Concerning the effect on ^{3}H-AA liberation from membrane phospholipids, CQ was more effective on platelets stimulated with thrombin as compared with platelets

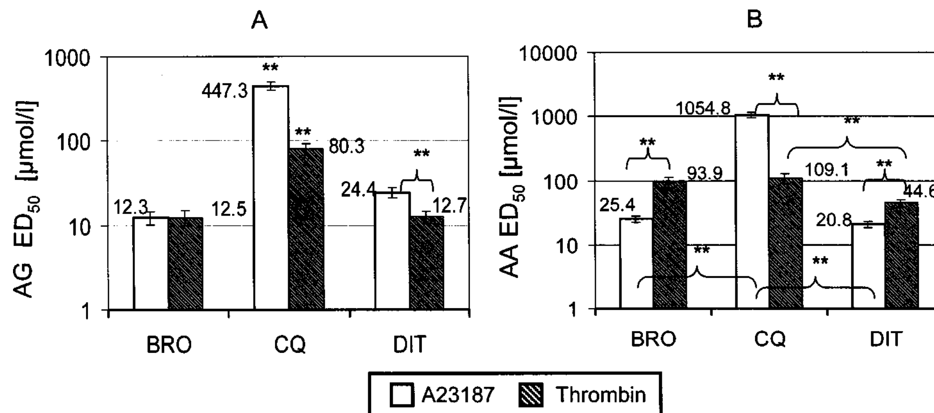

Fig. 5. Comparison between the effect of bromadryl (BRO), chloroquine (CQ) and dithiaden (DIT) by means of ED_{50} values on inhibition of aggregation (panel A) and $cPLA_2$ activation (expressed by means of arachidonic acid-AA liberation — panel B) in isolated human blood platelets stimulated with thrombin and A23187. n = 4 to 6±SEM, ** p≤0.01.

stimulated with A23187 (panel B). In the highest concentration used (1000 µmol/l), CQ decreased the AA liberation from A23187-stimulated platelets only by 5 %.

The effect of BRO, DIT and CQ on the aggregability and ^{3}H-AA liberation from platelet membrane phospholipids as expressed by ED_{50} values is compared in Figure 5. ED_{50} values for the inhibition of aggregation are given on panel A and are very close for BRO and DIT and for both stimuli, thrombin and A23187. A significant difference was found for DIT in ED_{50} values. A double concentration of DIT is required to inhibit the effect of calcium ionophore A23187 as compared with inhibition of thrombin (24.4 against 12.7 µmol/l).

Comparatively much higher concentrations of CQ are needed to reach E_{50} values. For A23187-stimulated platelets a concentration of 447 µmol/l, for thrombin-stimulated platelets 80 µmo/l of CQ are to be used.

As compared with inhibition of aggregation, significantly higher concentrations of BRO and DIT are necessary for inhibition of thrombin stimulated ^{3}H-AA liberation (Fig. 5, panel B). The concentration ratio evaluated for BRO is 25.4:93.9 µmol/l, for DIT 20.8:44.6 µmol/l, both in favour of A23187-stimulation. On the other hand, an opposite ratio was found for CQ-pretreated and thrombin stimulated platelets, yet the ED_{50} values were much higher: 1054.8:109.1 µmol/l.

Discussion

The cationic amphiphilic drugs tested, i.e. alprenolol, metipranolol, propranolol, bromadryl, dithiaden and chloroquine, inhibited aggregation of blood platelets in vitro. Inhibition of aggregation by drugs from different pharmacological groups correlated with their physico-chemical properties, particularly with liposolubility and with the ability to fluidise platelet membrane phospholipids (24, 25). Atenolol, oxprenolol and practolol with low partition coefficients and no effect on membrane fluidisation, had a very low or no effect on inhibition of platelet aggregation in vitro. Dose-dependent inhibition of platelet aggregation by CAD also correlated with their inhibition of membrane phospholipid peroxidation (as measured by means of malondialdehyde), particularly with inhibition of thromboxane generation in the same platelets. The same was found for rat and human platelets (17, 28, 30, 32). It is evident from these results that the inhibitory effect of CAD was similar whether platelets were stimulated at membrane receptor site (thrombin) or with stimuli bypassing membrane receptors (Ca^{2+} ionophore A23187). Consequently, both stimuli elevate intracellular Ca^{2+} necessary for platelet activation (4). It was demonstrated that inhibition of aggregation significantly correlated with the inhibition of thromboxane generation as well as with the inhibition of AA liberation in the same platelets (15, 17, 26, 27). Since the CAD investigated had no relationship to platelet cyclooxygenase, as shown for non-steroidal antiinflammatory drugs, we paid attention to the initial enzyme of platelet arachidonate cascade, i.e. phospholipase A_2.

Platelet cytosolic phospholipase A_2 ($cPLA_2$) crucially participated in hydrolytic splitting of AA from membrane phospholipids. In this respect the hydrolytic effect of diacylglycerol lipase and phospholipase C is considered negligible (13, 22). As a measure of activation/inhibition of platelet $cPLA_2$ the amount of AA split by hydrolysis from membrane phospholipids is considered (13, 22).

Pharmacological inhibition of PLA_2 is a matter of intensive investigation (39). Considering the existence of three forms of PLA_2 and a number of subgroups of enzymes in each of it (34), a selective approach is required for the inhibition of a particular target enzyme.

Much evidence is available for inhibition of secretory PLA_2($sPLA_2$). CAD with lysosomotropic activity inhibited $sPLA_2$ at intracellular level. Two possible inhibitory mechanisms have been suggested. First, binding of CAD to an active centre of enzyme with its subsequent inactivation. Second, binding of CAD

to membrane phospholipid, thus forming a non-hydrolysable complex for AA (9, 14, 18). At present attention is paid to pharmacological inhibition of PLA_2 at extra- and intracellular levels. In general, there is no true inhibition but neutralisation of AA metabolites. The first group is represented by ExPLis drugs (extracellular PL_s inhibitors) (39) like free radical scavengers, PAF antagonists, enzyme inhibitors or polymeric carriers with phosphatidylethanolamine (2, 5, 8, 23, 35, 38).

The second group of drugs indirectly affecting $sPLA_2$ at intracellular level is represented by nonsteroidal antiinflammatory drugs (inhibitors of cyclooxygenase) and inhibitors of lipooxygenase (12, 36).

The inhibition of $cPLA_2$ can take place only at intracellular level. Enucleated blood platelets are specific in this matter. Inhibition of platelet $cPLA_2$ with highly lipophilic CAD (alprenolol, metipranolol, propranolol, bromadryl, dithiaden, chloroquine) suggest their transmembrane movement intracellularly and subsequent interference with platelet $cPLA_2$ and then aggregation. Propranolol and chloroquine at therapeutical levels were demonstrated to accumulate in blood platelets (3, 37). Considering their physico-chemical properties, the same might be true for BRO and DIT (16).

From the inhibition of stimulated AA liberation and subsequent inhibition of aggregation it is suggested that cytosolic PLA_2 should be the site of action for the CAD investigated. Yet the level and mechanism of action by which CAD interact with $cPLA_2$ remain to be investigated. Individual steps participating in $cPLA_2$ activation, like intraplatelet calcium mobilisation, induction of phospholipase C(PLC) cascade or proteinkinase C(pKC) activation and subsequent phosphorylation, must be considered (4, 33).

The CAD investigated do not represent true antiplatelet drugs as they belong to different pharmacological groups. Nevertheless, their antiplatelet activity depending on the chemical structure and physico-chemical properties provides important evidence for target interference with activated platelets.

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