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Anti-inflammatory and platelet anti-aggregant activity of phospholipase-A_2 inhibitors

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Mepacrine, papaverine, *p*-bromophenacyl bromide and 2,3-dibromo(4′-cyclohexyl-3′-chloro)-phenyl-4-oxo-butyric acid (CB 874) inhibit the hydrolysis of phospholipids induced by thrombin in dog platelets. They also exhibit anti-inflammatory and anti-aggregant properties. These biological activities may be explained by a direct or indirect inhibitory action on phospholipase A_2. Phospholipase A_2 inhibitors may block not only the release of arachidonic acid and its subsequent conversion into prostaglandins but also the formation of lysophospholipids involved in inflammation and/or platelet aggregation.

Since the observations of Gryglewski et al (1975) studies have confirmed that anti-inflammatory corticosteroids inhibit phospholipase-A_2 (PL-A_2) activity, for example in mouse fibroblasts culture (Hong & Levine 1976), in guinea-pig perfused sensitized lungs (Nijkamp et al 1976) and in inflammatory proliferative skin disease (Hammarström et al 1977). It is also well established that PL-A_2 is activated by mechanical trauma, antigen challenge or injection of rabbit aorta contracting substance releasing factor or histamine (Blackwell et al 1978), all these conditions being associated with inflammation. Other work also shows that, during platelet aggregation, PL-A_2 is activated by either collagen or thrombin (Blackwell et al 1978) and by calcium ionophore A23187 (Pickett et al 1977). It thus appears that PL-A_2 may play a key role in inflammatory and aggregation processes. If this hypothesis holds true, inhibitors of PL-A_2 should then have therapeutic potential. In the present work we have attempted to substantiate this hypothesis by testing the anti-inflammatory and platelet anti-aggregatory activities of various agents exhibiting an inhibitory action on PL-A_2.

MATERIALS AND METHODS

Compounds. The following drugs and chemicals were used: [1-^{14}C]arachidonic acid (50 mCi mmol^{-1}-NEN), [1-^{14}C]oleic acid (60 mCi mmol^{-1}, Amersham), silicic acid (Mallinckrodt, 100 mesh), carrageenan (Viscarin, Marine Colloids), thrombin (Hoffman La Roche), Phospholipase-A_2 from *Vipera russelli* (Sigma), mepacrine (Quinacrine-Specia), *p*-bromophenacyl bromide (BPB) (Sigma), calcium ionophore A 23187 (generously supplied by Eli Lilly), 874 CB: 2,3-dibromo (4′-cyclohexyl-3′-chloro)phenyl-4-oxo-butyric acid.

Preparation of platelet rich plasma (PRP). Dogs were anaesthetized (pentobarbitone 30 mg kg^{-1} i.p.). Blood was withdrawn from a catheter placed in the femoral artery and collected into one tenth volume of sodium citrate (3·8% w/v). The blood was centrifuged at 300 *g* for 15 min and the PRP removed.

Evaluation of PL-A_2 inhibiting potency on PL-A_2 from activated platelets. The labelling of the platelets, incubation procedures and lipids extraction were carried out according to Bills et al (1976).

Evaluation of PL-A_2 inhibiting potency on PL-A_2 from Vipera russelli. The substrate 2-([1-^{14}C]-oleyl)-phosphatidylcholine was prepared as described by Blackwell et al (1977).

Assay procedure. 0·06 μg of PL-A_2 (10·6 U mg^{-1} of protein) was dissolved in 2 ml of phosphate buffer (0·1 M, pH 6·5 and 10^{-3} M in $CaCl_2$) with or without the inhibitors. After 15 min of preincubation (37° C) the substrate (100 000 d min^{-1}) was added and the reaction continued for 10 min after which it was stopped by 4·5 ml of EDTA 0·1 M. The incubation medium was extracted with 10 ml of chloroform–methanol (1:1). The aqueous phase was extracted again with 8 ml of chloroform–methanol (5:1) after acidification with 400 μl of formic acid. The two extracts were pooled, evaporated to dryness under nitrogen and treated for lipid separation as used above for platelets.

Biological tests

Inflammation. The anti-inflammatory activity of the various compounds was assessed by their effect on carrageenan-induced rat paw oedema (Winter et al 1963).

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Platelets aggregation. Aggregation, recorded turbidimetrically (Born 1962), was carried out with washed rabbit platelets resuspended in Tyrode solution (Vargaftig et al 1975).

RESULTS

Activation of PL-A_2 in platelets

A comparison of the three lipid fractions, e.g. phospholipids, arachidonic acid and prostaglandins obtained from thrombin-treated vs untreated platelets (control) showed (Table 1) that thrombin caused a decrease of the phospholipid pool and a large increase of both arachidonic acid release (fourfold) and prostaglandins synthesis (tenfold). The most likely explanation is the activation of Pl-A_2 by thrombin which results in an enhancement of platelet phospholipids hydrolysis, yielding free arachidonic acid.

Table 1. Action of thrombin on phospholipids hydrolsis, arachidonic acid mobilization and subsequent prostaglandins synthesis in platelets. Platelets were labelled by incubation of PRP with [^{14}C]arachidonic acid. After 1 h, excess radioactivity was washed out. The labelled platelets were then suspended in a Tris-buffer solution (pH 7·4) with or without thrombin (5 U ml^{-1} test solution). After 5 min, lipids were extracted and separated on columns of silicic acid. The fraction containing respectively free fatty acids, prostaglandins and phospholipids were isolated and counted for radioactivity. Results are expressed as percentage of total counts of the three fractions. Each value is the mean ± s.e.m. of 14 experiments.

Fraction	Control	Thrombin 5 U ml^{-1}
Phospholipids	97·50 ± 0·25	84·22 ± 0·56**
Arachidonic acid	1·82 ± 0·16	7·08 ± 0·45**
Prostaglandins	0·68 ± 0·11	8·70 ± 0·28**

** Significantly different from control at $P<0·01$.

A comparable activation of PL-A_2 can be obtained with calcium ionophore A 23187 (Table 2).

Table 2. Action of A 23187 on phospholipids hydrolysis, arachidonic acid mobilization and subsequent prostaglandins synthesis in platelets. Results are expressed as percentage of total counts of the three fractions. Mean ± s.e.m. of 4 experiments. For method see Table 1.

Fraction	Control	A 23187 (10^{-6} M)
Phospholipids	98·28 ± 0·03	81·55 ± 0·19**
Arachidonic acid	1·14 ± 0·01	10·63 ± 0·39**
Prostaglandins	0·58 ± 0·01	7·82 ± 0·22**

** Significantly different from control at $P<0·01$.

Inhibition of PL-A_2 in thrombin activated platelets

Papaverine and mepacrine provoked a dose-dependent inhibition of the hydrolysis of phospholipids and of the release of arachidonic acid from dog platelets activated by thrombin (Fig. 1). They were potent inhibitors of the activation of PL-A_2. Their action resulted in a decrease of prostaglandin synthesis. *p*-Bromophenacyl bromide (BPB), although less potent, nevertheless had the same pattern of action.

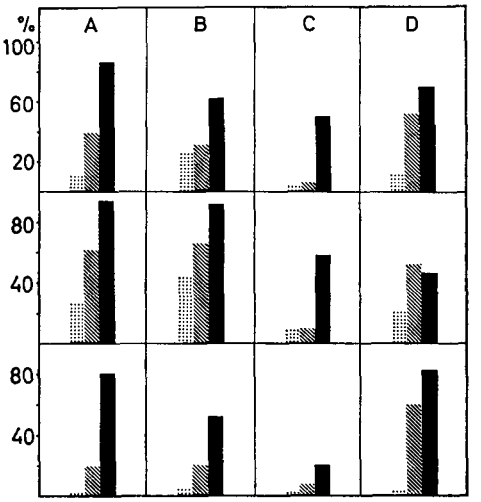

FIG. 1. Action of papaverine (A), mepacrine (B), BPB (C) and CB 874 (D) on phospholipids hydrolysis (top), arachidonic acid (middle) and prostaglandins synthesis (bottom) on dog platelets activated by thrombin (for method see Table 1). All drugs have been studied at three concentrations: 10^{-6} M stippled columns, 10^{-5} M hatched columns, 10^{-4} M solid columns. Results are expressed in percentage inhibition of values obtained with thrombin alone (5 U ml^{-1}). Standard errors of the means (n = 4) are less than 10%.

CB 874 was also a potent inhibitor of phospholipid hydrolysis but it had a more marked action on prostaglandin synthesis than the other three compounds as shown by a comparatively weaker effect on the arachidonic fraction and a stronger effect on the prostaglandin fraction.

Inhibition of PL-A_2 in A 23187 activated platelets

The PL-A_2 inhibitory activities of the four compounds were tested on platelets activated by the calcium ionophore A 23187 (Fig. 2). Neither papaverine nor mepacrine produced any inhibitory effect while both BPB and CB 874 were potent inhibitors of PL-A_2 as with thrombin-activated platelets. Therefore we studied these four com-

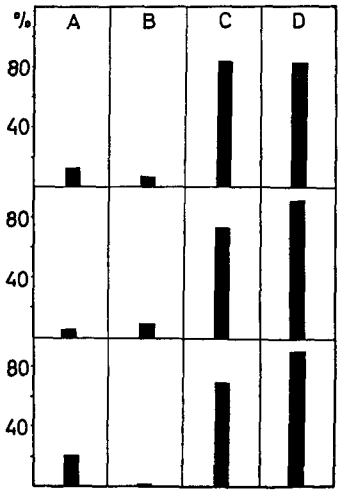

FIG. 2. Action of papaverine (A), mepacrine (B), BPB (C) and CB 874 on phospholipids hydrolysis (top) arachidonic acid release (middle) and prostaglandins synthesis (bottom) on dog platelets activated by A 23187 (for method see Table 1). All drugs have been studied at 10^{-4} M. Results are expressed in percentage inhibition of values obtained with A 23187 alone (10^{-6} M). Standard errors of the means (n = 4) are less than 10%.

pounds on a purified PL-A_2 to distinguish between an inhibition of PL-A_2 activation and a direct inhibition of PL-A_2.

Inhibition of PL-A_2 from Vipera russelli

We compared the inhibition by papaverine, mepacrine, BPB and CB 874 of the hydrolysis of 2-([1-^{14}C]-oleyl)phosphatidylcholine by PL-A_2 from *Vipera russelli* (Fig. 3). Papaverine and mepacrine were inactive, but BPB and CB 874 were

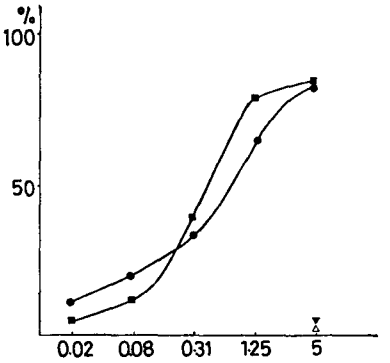

FIG. 3. Inhibition of the hydrolysis of 2-([1-C^{14}]oleyl) phosphatidyl choline by PL-A_2 from *Vipera russelli* with BPB (●), CB 874 (■), papaverine (▲) and mepacrine (△). Results (mean of two assays) are expressed as percentage inhibition (ordinate) of the release of [1-^{14}C] oleic acid by PL-A_2 alone. Abscissa: concentration ($\times 10^{-5}$ M).

true inhibitors. The IC50 values were respectively 9×10^{-6} and 5×10^{-6} M.

Biological activity of papaverine, mepacrine, BPB and CB 874

The anti-inflammatory activity of these compounds was evaluated in rats with carrageenan-induced paw oedema. Mepacrine 100, BPB 25, and CB 874 10 mg kg^{-1} i.p. had significant anti-inflammatory activity (Fig. 4). Papaverine 10 mg kg^{-1} i.p. was not active. Indomethacin, used as a reference, was active at 3 mg kg^{-1}.

The assays of the anti-aggregant capacity were carried out on washed rabbit platelets. All four compounds inhibited thrombin-induced platelet aggregation (Fig. 5); BPB was the most potent,

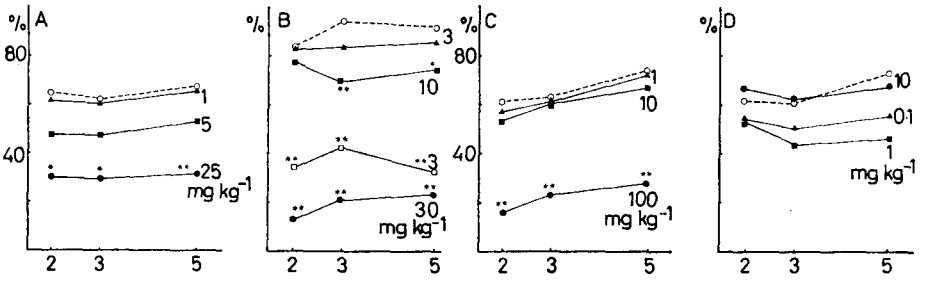

FIG. 4. Action of BPB (A), CB 874 (B), mepacrine (C) and papaverine (D) on carrageenan-induced oedema in the rat paw. Male rats (108 g) were treated i.p. with the drugs. One h later they received a 50 µl injection of a 1% carrageenan solution into the plantar tissue of the hind paw. The intensity of the resulting oedema was measured by plethysmometry, 2, 3 and 5 h after the injection of carrageenan. Results are expressed as percentage of the increase of the paw volume (ordinate). The doses of drugs (mg kg^{-1}) administered i.p. are indicated. The symbol (○) corresponds to control values and (□) to indomethacin used as reference. Each point represents the mean of 10 experiments. Abscissa: time (h).

* Significantly different from control ($P<0.05$).

** $P<0.01$.

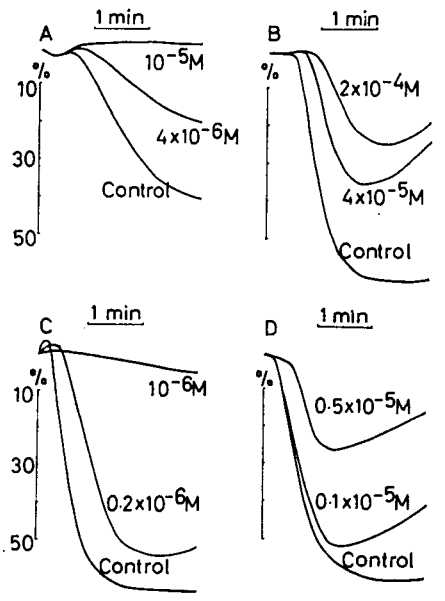

FIG. 5. Action of papaverine (A), mepacrine (B), BPB (C) and CB 874 (D) on the platelet aggregation induced by thrombin (0·005 Uml^{-1}). Washed rabbit platelets suspended in tyrode buffer were used. Drugs were added 15 min (BPB) or 5 min (for others) before thrombin at final concentrations indicated on the figure. Ordinate: light transmission (%). Abscissa: time (min).

with 100% inhibitory action at 10^{-6} M. For papaverine 100% inhibition was at 10^{-5} M and CB 874 had a similar potency but mepacrine was much less effective, 50% inhibition was obtained at about 10^{-4} M.

DISCUSSION

Our aim was to determine if inhibition of phospholipase A_2 activity by agents others than corticoids resulted in protection against inflammation and/or platelet aggregation. A search for such agents lead us to mepacrine, *p*-bromophenacyl bromide (BPB), CB 874 and papaverine. Their inhibitory effect on PL-A_2 was assessed by their ability to prevent or decrease the release of arachidonic acid from platelets in the presence of thrombin. While the four agents could not all be considered as true PL-A_2 inhibitors, they all reduced the release of arachidonic acid platelets. They did so, however, by different mechanisms. BPB and CB 874 were direct inhibitors of the enzyme since they inhibited the in vitro hydrolysis of 2-oleylphosphatidylcholine by the PL-A_2 of *Vipera russelli*. BPB selectively alkylates the histidine residue close to the active site of the enzyme, leading to its inactivation (Volwerk et al 1974). The mechanism of action of CB 874 is under investigation. Mepacrine, which was proposed as an inhibitor of PL-A_2 by Vargaftig & Dao Hai (1972), and papaverine, may be considered as indirect inhibitors; while they are unable to inactivate venom PL-A_2, they antagonize the PL-A_2 activation by thrombin in platelets. They would be expected to inhibit the PL-A_2 activation by calcium since they were without effect on PL-A_2 activated by ionophore A 23187.

The anti-inflammatory and/or anti-aggregant activities of these inhibitors of PL-A_2 were clearly demonstrated. The question arises of whether the pharmacological properties of these agents are the consequence of their PL-A_2 inhibiting activity or if other mechanisms are involved. The properties of papaverine can be explained by its inhibitory action on phosphodiesterase and the resulting increase in cAMP. But it is also possible that the action on cAMP promotes the calcium sequestration (Rasmussen & Goodman 1977) and decreases the free calcium necessary to PL-A_2 activation (Rittenhouse-Simmons & Deykin 1977).

The effects of BPB may be the consequence of a non-specific generalized action due to the alkylation of molecules other than PL-A_2.

Nevertheless Volwerk et al (1974) have demonstrated: (i) the alkylation of the histidine residue close to the active site by BPB is specific (ii) the alkylation by iodoacetic acid did not inactivate PL-A_2. Therefore BPB is probably not acting by alkylation and inactivation of enzymes related to inflammation and aggregation other than PL-A_2.

Papaverine, mepacrine, BPB and CB 874, among the few known PL-A_2 inhibitors have anti-inflammatory and/or anti-aggregant properties. The possible mode of action of these PL-A_2 inhibitors against inflammation and platelet aggregation requires further discussion. It can be explained by their dual action on phospholipids: (i) inhibition of arachidonic acid release, thus inhibition of prostaglandins synthesis (ii) inhibition of lysophospholipid production.

The role of prostaglandins in inflammation and aggregation has been well documented. Rather than PGE_2 and $PGF_{2\alpha}$, the determining factors in these processes appear to be PGG_2, and TXA_2 (Kuehl et al 1977). However, inflammation and platelet aggregation can still be produced in rats fed with a diet deprived of essential fatty acids, precursors of arachidonic acid (Bult & Bonta 1976; Bonta et al 1977). Moreover, Vargaftig (1977), has shown that thrombin and carrageenan still

cause aggregation while prostaglandin synthesis is blocked and that mepacrine and BPB inhibit this aggregation. The PL-A_2 inhibiting activity of these two agents may explain these findings. The inhibitory effect would decrease lysophospholipid formation. Recently the role of these compounds in platelet aggregation has been stressed by Bills et al (1977). These authors suggested that lysophospholipids are necessary in the sequence of membranal reactions that lead to the release reaction in the platelets. The importance of these lipid metabolites is also shown by the recent findings of Benveniste et al (1977) who found that platelet aggregating factor (PAF), a powerful aggregant agent, is a lysophospholipid.

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