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MULTIPLE EFFECTS OF A NEW ANTI-INFLAMMATORY AGENT, TIMEGADINE, ON ARACHIDONIC ACID RELEASE AND METABOLISM IN NEUTROPHILS AND PLATELETS

IAN AHNFELT-RØNNE and EDUARDO ARRIGONI-MARTELLI
Department of Pharmacology, Leo Pharmaceutical Products, DK-2750 Ballerup, Denmark

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Abstract—Casein-elicited rat peritoneal polymorphonuclear leukocytes (PMNL) and rabbit platelets were prelabelled with [1-^{14}C]arachidonic acid, and the effect of timegadine, a new anti-inflammatory agent, on the release and metabolism of arachidonic acid induced by A23187 (PMNL) and thrombin (platelets) was studied and compared with the effect of other compounds reported to affect these enzymatic mechanisms. Timegadine inhibited arachidonic acid release from both cells ($IC_{50} = 2.7 \times 10^{-5}$ M), the lipoxygenase activity in PMNL ($IC_{50} = 4.1 \times 10^{-5}$ M) and the cyclooxygenase activity in platelets ($IC_{50} = 3.1 \times 10^{-8}$ M). By these mechanisms, PMNL leukotriene B_4 formation was inhibited by 50% at 2.0×10^{-5} M, platelet thromboxane B_2 at 3.2×10^{-8} M, and platelet 12-HETE at 4.9×10^{-5} M. These effects might add to the understanding of the anti-inflammatory properties of timegadine.

Timegadine, *N*-cyclohexyl-*N''*-4-(2-methylquinolyl)-*N'*-2-thiazolylguanidine, a new, basic non-steroidal anti-inflammatory drug (NSAID) [1, 2], is a potent inhibitor of microsomal prostaglandin synthetase from bovine seminal vesicles and other tissues, and a relatively weak inhibitor of horse and rabbit platelet lipoxygenase [3].

Platelet lipoxygenase transforms arachidonic acid to 12-L-hydroxyeicosatetraenoic acid (12-HETE) [4], which is chemotactic for polymorphonuclear leukocytes (PMNL) [5] and macrophages [6]. A much more potent chemotactic agent is, however, leukotriene B_4 [7], which is synthesized from arachidonic acid by a lipoxygenase present in leukocytes [8]. Leukotrienes are released in response to immunologic stimulation [9] and may play an important role in immunologically mediated diseases, such as rheumatoid arthritis.

As both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism lead to the formation of putative mediators of inflammation, dual inhibitors of these pathways may have some advantages over 'aspirin-like' drugs in the treatment of inflammatory disease [10]. In addition, compounds that reduce the formation of those metabolites through inhibition of the arachidonic acid releasing enzyme, phospholipase A_2, may also show anti-inflammatory activity [11].

Many cyclooxygenase inhibitors are known (for review, see [12]). In contrast, few compounds inhibiting leukotriene formation have been described. These include [9] the antioxidants nordihydroguaiaretic acid (NDGA) and BW755C, a phenidone derivative; further, the tetraynoic analogue of arachidonic acid, ETYA, and, at high concentrations, the NSAIDs benoxaprofen, indomethacin and diflunisal. 15-HETE has also been reported to inhibit leukotriene biosynthesis [13]. Non-steroidal inhibitors of phospholipase A_2 activity include mepacrine and bromphenacyl bromide [14], indomethacin, sodium flufenamate and meclofenamate [15], and local anaesthetics, chlorpromazine and propranolol [16].

PMNL mainly release arachidonic acid and leukotrienes when exposed to the calcium ionophore A23187 [17]. Platelets mainly release arachidonic acid, thromboxanes and 12-HETE when exposed to thrombin [18]. We have prelabelled PMNL and platelets with radioactive arachidonic acid and triggered its release and metabolism with those agents in the presence of timegadine in order to study possible effects of this new anti-inflammatory compound on arachidonic acid turnover in intact, inflammatory cells. The results of these experiments are reported.

MATERIALS AND METHODS

PMNL. Peritoneal exudates containing more than 85% PMNL were elicited in female Sprague–Dawley rats, weighing 125–150 g, by injection of sodium caseinate [19]. The cells were harvested after 20 hr by injection of 20 ml Hank's balanced salt solution (GIBCO, catalogue No. 402S) containing 12.5 U/ml heparin, and washed twice by centrifugation (200 *g*, 10 min) and resuspension in 134 mM NaCl, 5 mM glucose, 15 mM Tris–HCl buffer, pH 7.4 (buffer A) containing 0.5% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO). The cells were labelled with radioactive arachidonic acid by incubation in 0.5% BSA–buffer A at 30° for 90 min with 5 μCi [1-^{14}C]arachidonic acid, 58.4 mCi/mmole (The Radiochemical Centre, Amersham, U.K.). Excess arachidonic acid was removed by washing twice in

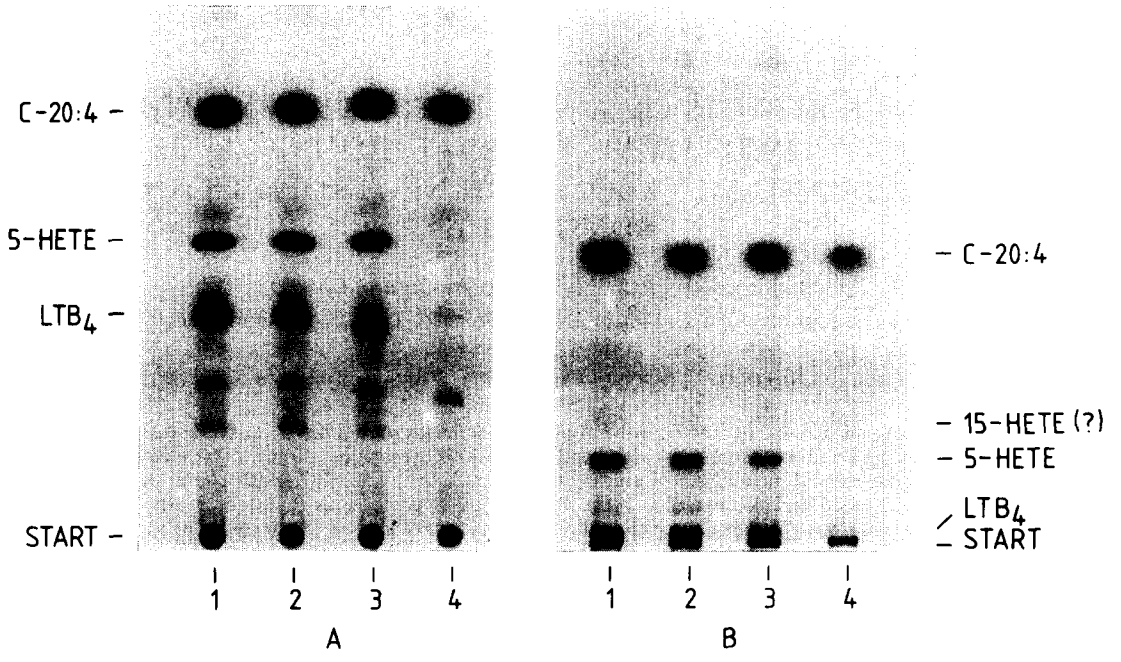

Fig. 1. Autoradiograms showing TLC separations in solvent system A (left) and B (right) of radioactive compounds released from [1-^{14}C]arachidonic acid-labelled rat peritoneal PMNL challenged *in vitro* with A23187 in the presence (2–4) or absence (1) of timegadine. Drug concentrations were 10^{-6} M (2), 10^{-5} M (3) and 10^{-4} M (4). C-20:4: arachidonic acid.

5 ml 0.5% BSA–buffer A, and the PMNL were finally resuspended in buffer A without BSA at 5×10^6 cells/ml. The PMNL suspension (475 μl) was preincubated with 5 μl drug solution in DMSO or water (mepacrine) for 5 min at 37°. Control tubes contained the same amount of DMSO or water. Equal volumes of 10^{-3} M A23187 (CalBiochem, San Diego, CA) in ethanol and 0.5 M $CaCl_2$ in buffer A were mixed, and 20 μl of this solution was added to the cell suspension to give a final concentration of 2×10^{-5} M A23187 and 8×10^{-3} M $CaCl_2$. After 5 min incubation at 37°, the tubes were chilled on ice and the cells were precipitated at 3000 *g*, 10 min. The supernatants were extracted twice with 2 ml ethyl acetate containing 1 μg/ml arachidonic acid as carrier, then titrated to pH 3 and extracted twice with 2 ml ethyl acetate. The extracts were combined, evaporated to dryness *in vacuo* and the residues dissolved in methanol and separated on 0.25 mm silica-coated aluminium sheets (E. Merck, Darmstadt, F.R.G.) in the solvent mixture chloroform–methanol–acetic acid–water (90:9:1:0.65) (solvent A). For the purpose of identifying the radioactive compounds released from PMNL, the extracts were separated on 0.25 mm silica-coated glass plates fitted with a polar concentrating zone (E. Merck) in the solvent mixture petroleum ether–diethyl ether–acetic acid (50:50:1) (solvent B) as described by Vanderhoek *et al.* [13], and results similar to these authors' were obtained (Fig. 1). Three major radioactive compounds were released by PMNL. The least polar was identified as unmetabolized arachidonic acid by comparison with an authentic standard (Sigma). The identity of the more polar metabolites, which were not inhibited by 10^{-6} M indomethacin, was 5-HETE and LTB_4 as verified by analysis of the ultraviolet absorption spectra. 5-HETE showed an absorption peak at 235 nm characteristic of mono-HETEs with one pair of conjugated double bonds [20]. LTB_4 showed a major peak at 270 nm, and two minor peaks at 261 and 281 nm, characteristic of leukotrienes [21]. A minor spot, presumably 15-HETE [13], was not studied. Radioactive spots were located by autoradiography, cut out, eluted with methanol, mixed with ACS™ liquid scintillant (The Radiochemical Centre) and counted in a Beckman LS 200 liquid scintillation spectrometer.

Platelets. Rabbit platelet-rich plasma (PRP) was prepared as previously described [3]. PRP from 20 ml blood was labelled with [1-^{14}C]arachidonic acid by incubating with 0.5 μCi of this radiochemical for 90 min at 30°. Platelets were then washed three times in buffer A containing 10^{-3} M EDTA, and finally resuspended in 0.25% BSA–buffer A at 10^7 cells/ml. The platelet suspension was preincubated with drugs as described above, and exposed to 4 U/ml thrombin (Leo Pharmaceutical Products, Ballerup, Denmark) and 4×10^{-3} M $CaCl_2$ (final concentration) for 20 min at 37°. The supernatants were extracted and analysed as described for PMNL. Four major radioactive spots were present (Fig. 4), and these were identified as arachidonic acid, 12-HETE, 12-hydroxyheptadecatrienoic acid (HHT) and TXB_2 by comparison with authentic standards, and by methods previously described [22].

Calculations. The formation of an arachidonic acid metabolite is determined by (1) substrate availability, and (2) activity of the specific metabolic enzyme. A drug-induced reduction of the actual amount of metabolite formed may be the result of (1) a decrease in substrate availability (by inhibition of phospholipase A_2), (2) an inhibition of the metabolic enzyme,

or (3) a combination of both. Calculation of the total amount and distribution of radioactivity released allows discrimination between those mechanisms.

Let A be the radioactivity (in dpm) associated with arachidonic acid released in absence of drugs, and M a metabolite. Let the same parameters in presence of a drug be a and m. The total release of radioactivity is $T = A + \Sigma M$, and $t = a + \Sigma m$.

Inhibition of specific enzyme activities is calculated by the following formulae:

(1) phospholipase A_2 (i.e. release of radioactivity): $1 - t/T$;
(2) specific metabolic enzymes (e.g. lipoxygenase): $1 - (m/M \times T/t)$;
(3) inhibition of the actual formation of a specific metabolite is $1 - m/M$.

IC_{50} was calculated by computerized linear regression analysis of the concentration vs inhibition curve.

Drugs. Timegadine and acetylsalicylic acid were obtained from Leo Pharmaceutical Products, indomethacin from Dumex A/S (Denmark), NDGA from Sigma, BW755C from The Wellcome Foundation (U.K.), benoxaprofen from E. Lilly (U.K.), mepacrine from Mecobenzon A/S (Denmark) and naproxen from Astra-Syntex (Sweden).

RESULTS

PMNL experiments

The distribution of radioactivity released by A23187 from PMNL prelabelled with [1-^{14}C]arachidonic acid is shown in autoradiograms of TLC-analysed extracts (Fig. 1). The relative amounts of radioactivity associated with the different metabolites in control assays were: arachidonic acid 40.6 ± 4.3%, 5-HETE 24.2 ± 1.2%, and LTB_4 35.3 ± 3.3% (mean ± S.E.M., $n = 16$). Fig. 1 also shows the concentration dependent inhibitory effects of timegadine on 5-HETE and LTB_4 formation. The decreased formation of these products was not accompanied by an increased formation of other metabolites and/or by greater amounts of free arachidonic acid. These findings suggest that timegadine reduces the release of total radioactivity from ionophore stimulated PMNL, likely through an inhibition of phospholipase A_2 activity. Table 1 shows the

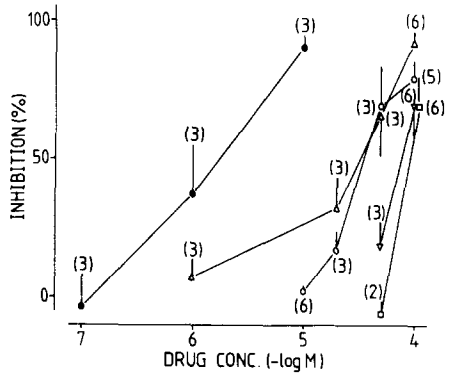

Fig. 2. Inhibition of rat peritoneal PMNL lipoxygenase. Cells prelabelled with [1-^{14}C]arachidonic acid were preincubated with drugs for 5 min prior to addition of A23187. Symbols used: timegadine (○), NDGA (●), BW755C (△), indomethacin (▽) and benoxaprofen (□). Each point shows the mean value of several experiments. The number of experiments are shown in brackets, and vertical bars represent S.E.M.

inhibitory potency of timegadine and several other compounds on these enzymatic activities, and on the actual formation of leukotriene B_4. As far as lipoxygenase inhibition is concerned, the order of potency was NDGA > BW755C ≥ timegadine > benoxaprofen ≥ indomethacin. Mepacrine, acetylsalicylic acid and naproxen did not show any inhibition of this pathway. None of the inhibitors showed any preferential inhibition of one lipoxygenase metabolite over another, which indicates an inhibition of the first step of lipoxygenase activity, i.e. formation of 5-HPETE. With the exception of NDGA, the inhibitors showed quite steep slopes of the concentration vs inhibition curves (Fig. 2). In order to exclude the possibility of cell-death as the cause of this abrupt inhibition the viability of cells after 5 min exposure to the different drugs at 10^{-4} M was tested by the dye exclusion method, and only BW755C was found to be slightly toxic at this concentration (viability = 77%; all other drugs >93% of the control).

Timegadine was about three times as potent as indomethacin and benoxaprofen in reducing the

Table 1. Drug effects on enzyme activities involved in the release and metabolism of arachidonic acid, and on the actual formation of leukotriene B_4, in casein-elicited rat peritoneal PMN leukocytes prelabelled with [1-^{14}C]arachidonic acid and challenged with the calcium ionophore A23187

		Inhibition* of: Enzyme activities		Formation of:
Drug	N	Phospholipase A_2	5-Lipoxygenase	Leukotriene B_4
Timegadine	3	$(2.7 \pm 0.3) \times 10^{-5}$	$(4.1 \pm 1.7) \times 10^{-5}$	$(2.0 \pm 0.3) \times 10^{-5}$
Benoxaprofen	4	$(9.1 \pm 4.0) \times 10^{-5}$	$(8.7 \pm 4.4) \times 10^{-5}$	$(5.2 \pm 1.2) \times 10^{-5}$
BW755C	4	$>10^{-4}$	$(3.1 \pm 1.8) \times 10^{-5}$	$(2.8 \pm 0.6) \times 10^{-5}$
NDGA	3	$>10^{-4}$	$(2.0 \pm 1.9) \times 10^{-6}$	$(1.5 \pm 0.9) \times 10^{-6}$
Indomethacin	3	$(8.4 \pm 4.3) \times 10^{-5}$	$(9.7 \pm 3.8) \times 10^{-5}$	$(5.0 \pm 1.2) \times 10^{-5}$
Naproxen	3	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
Aspirin	3	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
Mepacrine	5	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$

* IC_{50} (M). Values are means ± S.D. N = number of experiments.

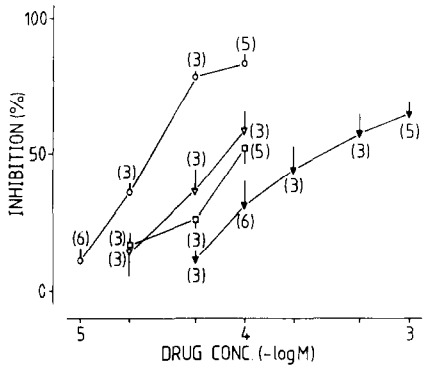

Fig. 3. Inhibition of rat peritoneal PMNL phospholipase A_2. Drug effects on A23187 induced release of radioactivity from cells prelabelled with [1-^{14}C]arachidonic acid. Drugs shown are timegadine (○), indomethacin (▽), benoxaprofen (□) and mepacrine (▼). Other symbols, see legend to Fig. 2.

release of arachidonic acid from PMNL (Table 1). No other drugs showed activity in this respect up to 10^{-4} M. However, as mepacrine is known to be a phospholipase A_2 inhibitor this compound was tested at higher concentrations in order to validate the assay (Fig. 3), and an IC_{50} of $3.8 \pm 1.4 \times 10^{-4}$ M ($n = 5$) was calculated. The actual amount of LTB_4 released by PMNL exposed to ionophore depends on both substrate (i.e. arachidonic acid) availability and lipoxygenase activity. Inhibition of both phospholipase and lipoxygenase thus has a synergistic effect on LTB_4-synthesis as shown in Table 1. In fact, timegadine, benoxaprofen and indomethacin which inhibited both enzyme activities reduced the release of LTB_4, calculated as the actual amount of radiolabelled LTB_4 present in the medium, at lower concentrations than those needed to inhibit either enzyme. Table 1 only lists LTB_4, as this seems to be biologically the most important metabolite, but an equivalent inhibition of 5-HETE formation occurred. In contrast, the lipoxygenase inhibitors, BW755C and NDGA, reduced LTB_4 formation at concentrations similar to those inhibiting lipoxygenase activity.

Platelet experiments

Figure 4 shows the distribution of radioactivity released from prelabelled rabbit platelets triggered by thrombin in the absence or presence of timegadine. In the control tube the distribution was: arachidonic acid $27.7 \pm 3.2\%$, 12-HETE $34.3 \pm 2.9\%$, HHT $15.7 \pm 0.8\%$ and TXB_2 $22.2 \pm 1.6\%$ (mean ± S.E.M., $n = 15$). The products formed via the cyclooxygenase pathway, HHT + TXB_2, accounted for $37.8 \pm 2.4\%$ of the total radioactivity. Figure 4 shows a complete inhibition of the cyclooxygenase activity with 10^{-6} M timegadine. A concomitant increase of 12-HETE formation and of free arachidonic acid took place. However, at 10^{-4} M timegadine the release of arachidonic acid and the formation of various metabolites was almost com-

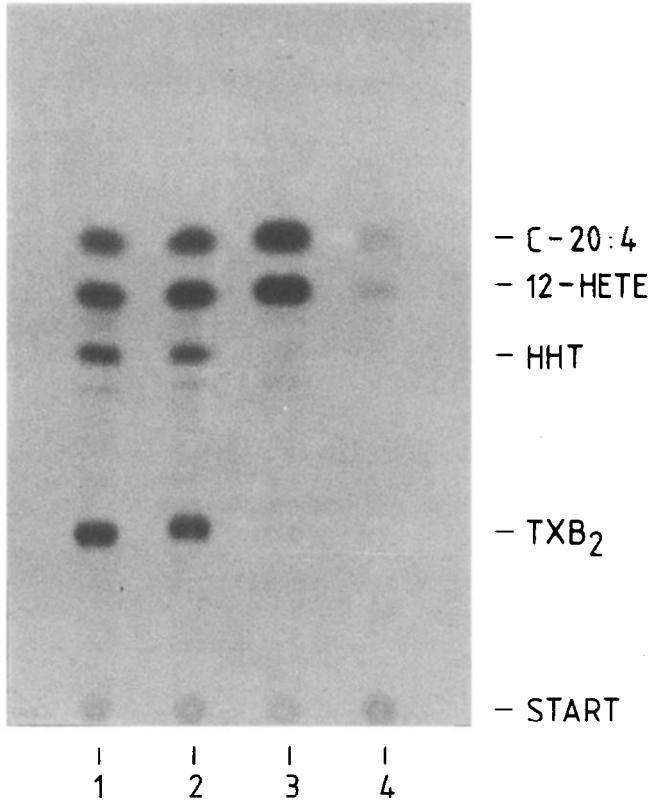

Fig. 4. Autoradiogram showing a TLC separation in solvent system A of radioactive compounds released from [1-^{14}C]arachidonic acid-labelled rabbit platelets challenged *in vitro* with thrombin in the presence (2–4) or absence (1) of timegadine. Drug concentrations were 10^{-8} M (2), 10^{-6} M (3) and 10^{-4} M (4). C-20:4: arachidonic acid.

Table 2. Drug effects on enzyme activities involved in the release and metabolism of arachidonic acid, and on actual formation of arachidonate metabolites, in rabbit platelets prelabelled with [1-^{14}C]arachidonic acid and challenged with thrombin

Drug	N	Inhibition* of: Enzyme activities			Formation of:	
		Phospholipase A_2	Cyclooxygenase	12-Lipoxygenase	Thromboxane B_2	12-HETE
Timegadine	5	$(2.7 \pm 1.3) \times 10^{-5}$	$(3.1 \pm 1.5) \times 10^{-8}$	$>10^{-4}$	$(3.2 \pm 0.9) \times 10^{-8}$	$(4.9 \pm 1.1) \times 10^{-5}$
Benoxaprofen	3	$>10^{-4}$	$(2.9 \pm 0.5) \times 10^{-5}$	$>10^{-4}$	$(2.9 \pm 0.2) \times 10^{-5}$	$>10^{-4}$
BW755C	5	$>10^{-4}$	$(1.6 \pm 0.9) \times 10^{-6}$	$(2.9 \pm 1.1) \times 10^{-5}$	$(1.9 \pm 0.4) \times 10^{-6}$	$(2.9 \pm 0.7) \times 10^{-5}$
NDGA	3	$>10^{-4}$	$(9.3 \pm 5.2) \times 10^{-6}$	$(7.1 \pm 2.9) \times 10^{-6}$	$(1.3 \pm 0.5) \times 10^{-5}$	$(1.3 \pm 0.5) \times 10^{-5}$
Indomethacin	5	$>10^{-4}$	$(9.4 \pm 7.4) \times 10^{-8}$	$>10^{-4}$	$(8.2 \pm 3.8) \times 10^{-8}$	$>10^{-4}$
Naproxen	3	$>10^{-4}$	$(1.9 \pm 1.2) \times 10^{-5}$	$>10^{-4}$	$(2.1 \pm 0.5) \times 10^{-5}$	$>10^{-4}$
Aspirin	3	$>10^{-4}$	$(1.1 \pm 1.4) \times 10^{-5}$	$>10^{-4}$	$(1.0 \pm 0.7) \times 10^{-5}$	$>10^{-4}$
Mepacrine	3	$(7.1 \pm 5.9) \times 10^{-5}$	$>10^{-4}$	$>10^{-4}$	$(3.2 \pm 0.7) \times 10^{-5}$	$(4.5 \pm 1.4) \times 10^{-5}$

* IC_{50} (M). Values are means ± S.D. N = number of experiments.

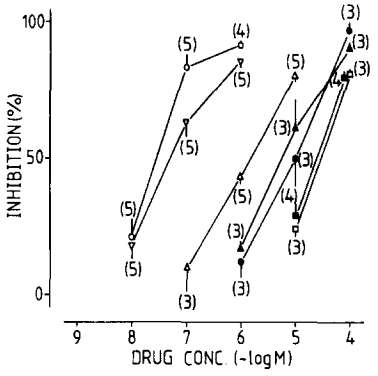

Fig. 5. Inhibition of rabbit platelet cyclooxygenase. Platelets prelabelled with [1-^{14}C]arachidonic acid were preincubated with drugs for 5 min prior to addition of thrombin. Drugs shown are timegadine (○), indomethacin (▽), BW755C (△), NDGA (●), aspirin (▲), benoxaprofen (□) and naproxen (■). Other symbols, see legend to Fig. 2.

pletely inhibited, thus suggesting a phospholipase A_2 inhibition. Only timegadine and mepacrine inhibited the thrombin-induced release of radioactivity at concentrations below 10^{-4} M (Table 2). Timegadine had an IC_{50} identical to that determined with PMNL (Table 1), and mepacrine was a 5 times more potent inhibitor of platelet than PMNL phospholipase, however, 3 times less potent than timegadine.

Timegadine and indomethacin inhibited the cyclooxygenase activity at nanomolar levels, timegadine being more potent than indomethacin. BW755C, NDGA, acetylsalicylic acid, naproxen and benoxaprofen, in that order of potency, inhibited cyclooxygenase at micromolar concentrations, and mepacrine was inactive up to 10^{-4} M (Table 2 and Fig. 5). Platelet lipoxygenase was only inhibited by NDGA and BW755C. Both timegadine and mepacrine, however, inhibited the release of 12-HETE (Table 2), and this was a result of the decrease in arachidonic acid availability.

DISCUSSION

The use of intact cells prelabelled with radioactive arachidonic acid to study the effect of timegadine on the formation of inflammatory mediators derived from this fatty acid has several advantages over the use of broken cell preparations. Firstly, it is possible to reveal effects on several different enzymes, such as phospholipase A_2, lipoxygenase and cyclooxygenase in a single assay. Secondly, the release of arachidonic acid from an endogenous pool is presumably more physiologically relevant than addition of exogenous arachidonic acid to cell suspensions; and finally, the ability of a drug to penetrate cell membranes and reach the target enzymes in a natural environment is an important aspect which can only be studied in intact cells.

In the present study we have used two different cells, both important in inflammatory reactions, to study the effect of timegadine: PMN leukocytes and platelets. In agreement with the results of others [8, 13] we found that PMNL mainly produce 5-HETE

and LTB_4, whereas platelets produce 12-HETE, HHT and TXB_2 [18]. Timegadine, a potent inhibitor of microsomal cyclooxygenase from various tissues [3], was found to be a very potent inhibitor of thromboxane synthesis from endogenous platelet arachidonic acid released by exposure to thrombin. Only indomethacin had comparable potency in this respect; the other NSAIDs and antioxidants used as reference compounds were several orders of magnitude less potent. It is generally agreed that inhibition of prostaglandin synthesis is an important aspect of antiinflammatory therapy, and the unusual potency of timegadine might partly explain the pharmacological profile of this drug [2]. Timegadine did not inhibit platelet lipoxygenase in the present investigation. This was surprising since we have previously shown an inhibition by this compound of the lipoxygenase activity in high speed supernatants of horse platelet homogenates, and even in intact rabbit platelets when the phospholipase was by-passed by incubating the cells in a medium containing free arachidonic acid [3]. Changes in experimental conditions might explain this discrepancy: if timegadine is a competitive inhibitor of lipoxygenase, as it is of cyclooxygenase [3], local concentrations of arachidonic acid, which are likely to be different in experiments using endogenous and exogenous substrate, will determine the IC_{50}. Furthermore, the use of 2.5 mg/ml albumin in the suspension medium of the prelabelled platelets might have shifted to the right the concentration–inhibition curve of timegadine, which is extensively protein-bound (M.P. Magnussen, unpublished observation); the earlier experiments were performed in a protein-free medium [3].

An important observation in the present study was the ability of timegadine to inhibit the phospholipase activity in both platelets and PMNL. In this respect timegadine was the most effective of the compounds tested, including mepacrine, a well-known phospholipase A_2 inhibitor [14]. Indomethacin has previously been reported to inhibit phospholipase A_2 obtained from human platelets and rabbit peritoneal PMNL with an IC_{50} within the range that we have obtained (Table 1) [15]. Other NSAIDs such as meclofenamic and flufenamic acid also inhibited phospholipase A_2 and the inhibition was a function of calcium concentration [15]. Timegadine, as a result of phospholipase A_2 inhibition, caused a 50% reduction in platelet 12-HETE formation at 5×10^{-5} M, and through simultaneous inhibition of PMNL phospholipase and lipoxygenase LTB_4 formation was reduced by 50% at 2×10^{-5} M. BW755C, NDGA, indomethacin and benoxaprofen have been reported to inhibit LTB_4 synthesis [9], and the present experiments confirmed the potencies of these drugs. We suggest that timegadine, with a potency similar to BW755C, may now be added to the list of *in vitro* leukotriene synthesis inhibitors. Future experiments will show whether timegadine inhibits LTB_4 formation *in vivo* which would add to the understanding of the pharmacology of this new anti-inflammatory compound.

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