Biochemical Pharmacology, Vol. 57, pp. 433–443, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00312-8

Anti-Inflammatory Mechanism of Alminoprofen: Action on the Phospholipid Metabolism Pathway

Céline Raguenes-Nicol, Françoise Russo-Marie,*† Gisèle Domage,‡ Nadia Diab,§ Egle Solito,* Fernand Dray,§ José L. Garcia Mace‡ and Gilles Streichenberger‡*

*INSERM U332, I.C.G.M., 75014 Paris; ‡Centre de Recherches des Laboratoires du Docteur E. Bouchara, 92000 Nanterre; and §Laboratoire de Biophysique, C.H.U. Cochin, 75014 Paris, France

ABSTRACT. Alminoprofen is a nonsteroidal anti-inflammatory drug (NSAID) of the phenylpropionic acid class. It has anti-inflammatory properties different from the classical NSAID. Using both *in vitro* systems of cells in culture and *in vivo* models of inflammation, we report here that alminoprofen possesses both antiphospholipase A_2 (PLA_2) activity and anti-cycloxygenase (COX) activity. The PLA_2 targeted by alminoprofen is likely the secretory phospholipase A_2 ($sPLA_2$) while the COX targeted is the COX-2. BIOCHEM PHARMACOL **57**;4: 433–443, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. PLA_2; melittin; alminoprofen; anti-inflammatory drugs; cyclooxygenase

Alminoprofen is a NSAID‖ of the phenylpropionic acid class. In human clinical practice, it is remarkably active against all inflammatory phenomena which combine pain and swelling (oedema). Pharmacological studies have demonstrated anti-exudative properties which distinguish the profile of the substance from that of other NSAIDs. The dissociation between the strong anti-inflammatory pharmacological activity of alminoprofen, identical to that of indomethacin in certain tests, and less potent anticyclooxygenase activity was first demonstrated by Fujiyoshi *et al.* [1]. This dissociation suggests that the anti-inflammatory action of alminoprofen could be exerted by mechanisms which differ from that of other NSAIDs, and in particular, by its ability to block the phospholipid metabolism pathway at an early step.

Among the complex mechanisms which lead to an inflammatory reaction, it is now known that inflammatory mediators, such as interleukin-1, tumor necrosis factor, bradykinin, and anaphylatoxins [2], act on specific receptors located on the surface of cells involved in the inflammatory reaction. After this ligand–receptor interaction, among the signalling pathways activated, two types of PLA_2, namely $sPLA_2$ and $cPLA_2$, are activated. Their activation elicits the production of arachidonic acid that is subsequently engaged into the production of prostaglandins and leukotrienes through further activation of specific enzymes, namely cyclooxygenases and lipoxygenases. These pharmacologically active lipid metabolites will in turn act in an autocrine and paracrine manner on specific receptors which may further lead to activation of phospholipase "s" A_2.

Cyclooxygenase activity is known to be the consequence of the activition of two iso enzymes (COX-1 and COX-2). Both enzymes use arachidonic acid as a substrate to form prostaglandins [3]. COX-1 is constitutive and is found in almost all tissues, and makes prostaglandins which protect the stomach and kidney from damage. COX-2, on the contrary, is induced by inflammatory stimuli such as cytokines during the inflammatory reaction and is found mostly in cells that respond to inflammatory mediators. It produces prostaglandins which contribute to the pain and swelling of inflammation. Most NSAIDs described up to now have been reported to inhibit both COX-1 and COX-2. Inhibition of COX-2 accounts for their anti-inflammatory effects and inhibition of COX-1 for their side-effects on the stomach and the kidneys.

Fujiyoshi *et al.* [4] showed that alminoprofen blocked the release of labelled arachidonic acid by mouse 3T3 fibroblasts, in a dose-dependent and statistically significant manner; this effect suggested an antiphospholipase A_2 activity. Their results led them to hypothesise that the anti-inflammatory mechanism of alminoprofen is based on inhibition of prostaglandin biosynthesis by an action on PLA_2 rather than on COX, which was poorly inhibited in an *in vitro* experiment using sheep seminal vesicles as a source of COX.

The objective of this work was to further analyse the

† Corresponding author: Dr. Françoise Russo-Marie, INSERM U332, ICGM, 22 rue Méchain, 75014 Paris, France. Tel. 33 140 46 04 11; FAX 33 140 51 77 49.

‖ *Abbreviations:* NSAID, nonsteroidal anti-inflammatory drug; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory PLA_2; COX, cyclooxygenase; BSA-FFA, fatty-acid free bovine serum albumin; EIA, Enzymatic immunoassay; RIA, Radioimmunological assay; FBS, fetal bovine serum; ASA, acetylsalicylic acid; PGE_2, prostaglandin E_2; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$, HUVEC, human umbilical vein endothelial cells; AACOCF3, arachidonyl trifluoromethyl ketone; DTT, dithiothreitol; PLA_2, phospholipase A_2; and DMEM, Dulbecco's modified Eagle's medium.

Received 29 January 1998; accepted 2 August 1998.

mechanism of action of alminoprofen by an *in vitro* study of the PLA_2 and COX activities of cells in culture and by an *in vivo* study of the inflammatory action of exogenous and endogenous PLA_2 in the rat. Our results suggest that alminoprofen possesses a dual anti-inflammatory action, by inhibiting both $sPLA_2$ but also COX-2.

MATERIALS AND METHODS

Reagents and Substances for In Vitro Studies

EGTA, folic acid, nonessential amino acids, BSA-FFA, dexamethasone, gentamicin, melittin from bee venom, quinacrine dihydrochloride, DTT, indomethacin, flurbiprofen, ASA, ionomycin, bradykinin, and phenylmethylsulfonyl fluoride were purchased from Sigma. Formic acid, ethanol, chloroform, gelatin, and methanol were purchased from Merck. [^{3}H]Arachidonic acid was obtained from NEN. Glutamine, DMEM, RPMI vitamins, and EDTA-trypsin were obtained from GIBCO. FBS was obtained from Boehringer. Assay kits for PGE_2 and 6-keto $PGF_{1\alpha}$ were purchased in the form of radioiodine kits from RIA Pasteur or in the form of EIA from Cayman Chemical. Alminoprofen was supplied by BOUCHARA. 5-Methane sulfonamido-6-(2,4-difluorothiophenyl)-1-indanone, L-745337, a COX-2 inhibitor, was a generous gift from Merck-Frosst Canada, Inc. The $cPLA_2$ inhibitor AACOCF3 was purchased from Calbiochem. The COX-1 enzyme (purified from ram seminal vesicles) was obtained from Spi-Bio and recombinant COX-2 from Calbiochem. The antibodies were either purchased (a polyclonal anti-COX-2 from BioMol Research Laboratories and a monoclonal anti-$cPLA_2$ from Santa Cruz Biotechnology) or raised in our laboratory in rabbits: polyclonal anti-COX-1 and anti-$sPLA_2$*.

Substances Used for in vivo *Studies*

A soluble exogenous PLA_2:PLA_2 extracted from *Naja mossambica mossambica* venom (Sigma) was used without specific purification [5]. An activator of endogenous PLA_2, melittin (Sigma), was also used without additional purification [6, 7] (PLA_2 impurity has been reported to be <20 units/mg solid). The following substances were also studied as to the reactions induced by these two calcium-dependent enzymes: alminoprofen; various anti-inflammatory drugs belonging to the same chemical class (arylcarboxylic derivatives ibuprofen, ketoprofen, diclofenac, and tiaprofenic acid); and anti-inflammatory drugs belonging to other chemical classes: indomethacin, ASA, oxicam (piroxicam), corticosteroids (dexamethasone), a nonspecific PLA_2 inhibitor, quinacrine, and a dual (cyclooxygenase and lipoxygenase) inhibitor, phenidone. All the drugs were from Sigma unless otherwise stated.

* Solito E, unpublished data.

Cell Cultures: HUVEC and the CRL1517 Cell Line of Human Skin Fibroblasts

The study was conducted in immortalised HUVEC on the CRL1517 cell line (human skin embryo fibroblasts purchased from the ATCC). Immortalisation of HUVEC was achieved by permanent transfection of a vector carrying the T gene of SV 40 under the control of a vimentin promoter and a neomycin resistance gene allowing selection [8]. All cell lines were cultured in DMEM containing 10% FBS and HUVEC were cultured in flasks previously covered with gelatin (0.2%). For subcultures, they were treated with EDTA-trypsin and subcultured at a 1:3 ratio. They were used almost at confluence for the experiments.

Western Blot Analysis

CRL1517 cells were collected in PBS containing 1 μM of phenylmethylsulfonyl fluoride, then spun at 150 *g* for 10 min. The pellet was resuspended in Tris–HCl pH 7.8. Proteins were extracted by freeze-thawing and extracts were centrifuged at 8000 *g* for 10 min. Fifty μg of total proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-rad). Immunostaining was performed using either rabbit polyclonal antibodies against COX-1 (dilution 1:1000), COX-2 (dilution 1:1000), and $sPLA_2$ (dilution 1:500) or mouse monoclonal antibody against $cPLA_2$ (dilution 1:1000). The primary antibodies were further detected using either donkey anti-rabbit or sheep anti-mouse antibodies conjugated to horseradish peroxidase (dilution 1:10,000). The labelled proteins were visualised with the enzymatic chemiluminescence detection system (Amersham).

Measurement of PLA_2 Activity

Endothelial cells or CRL1517 cells were incubated for one night in the presence of 0.1 μCi/mL/well of [^{3}H]arachidonic acid in culture medium containing 10% FBS. After incorporation, the medium was removed and each well was washed twice with 1 mL of DMEM containing no FBS but 0.25% BSA-FFA. The three supernatants were pooled and residual radioactivity measured (representing radioactivity not incorporated into the cells). Test substances were then put into FBS-free DMEM (+0.25% BSA-FFA) either under basal conditions or under stimulating conditions. Inhibitory agents were left in contact with cells for 20 min (except for dexamethasone, which was incubated for 8 hr), then incubated for a further 30 min either without any stimulating agent (basal conditions) or in the presence of a stimulating agent, melittin. At the end of this incubation period, the reaction was stopped by adding 300 μL of a cold solution containing 5 mM EGTA, 150 mM NaCl, and 1% BSA-FFA. The medium was removed, centrifuged for 5 min at 8000 *g*, and radioactivity was measured in an aliquot of 500 μL in a beta counter [9].

Measurement of In Vitro *COX-1 and COX-2 Activities*

Both enzymes used were purified either from ram seminal vesicles for COX-1 or as a human recombinant protein from *Spodoptera frugiperda* for COX-2. To prepare the cofactors necessary for the assay, the CRL 1517 cells were treated for 1 hr with 500 μM ASA. At the end of this incubation period, the microsomal fraction was prepared. Briefly, cells were washed with PBS and collected using a rubber policeman and centrifuged for 10 min at 300 *g*, then sonicated using a Branson sonicator (output 6, duty 60%). The homogenate was centrifuged for 10 min at 10,000 *g*, the pellet discarded, and the supernatant centrifuged again for 45 min at 200,000 *g*. The last pellet was recovered at 1 mg/mL of protein in Tris–HCl, 0.1 M, pH 7.4 and referred to as the microsomal fraction. The enzymatic reaction was carried out using the substrate: 25 μM arachidonic acid, the cofactors; 20 μg of the microsomal fraction and the enzyme, COX-1 (2U) or COX-2 (7U). The incubation was performed in the presence or absence of the tested inhibitors 5 μM indomethacin (for COX-1), 1 μM L745 (COX-2) or 50 μM alminoprofen. After a 30-min incubation period at 37°, the reaction was stopped by adding 1 mM ASA. The PGE_2 formed were measured using EIA as described below.

Measurement of Prostaglandins

Quantities of PGE_2 and of 6-keto $PGF_{1\alpha}$ were determined by RIA assay (RIA Diagnostics Pasteur) or by EIA (Cayman Chemical). When utilising the RIA, 10 μL of 5 N formic acid and 1 mL of methanol were added to the remaining volume of supernatant and the mixture was frozen for RIA. Prostaglandins were then extracted using a modified Bligh–Dyer method [10]. Chloroform (1.5 mL) was added to each tube. After centrifugation, the organic phase was removed. This stage was repeated and both organic phases were mixed and evaporated under nitrogen. At the end of the evaporation phase, 700 μL of RIA buffer was added. Extraction yields were determined using labelled standards. Quantities of PGE_2 and of 6-keto $PGF_{1\alpha}$ were then determined by RIA. When utilising the EIA, PGE_2 were measured directly on the supernatant without any previous separation.

Statistical Tests

MEASUREMENT OF **PLA_2** ACTIVITY. PLA_2 activity was determined by measuring radioactivity released by cells into the medium and normalised to the control. Values deviating by more than 15% from the mean replicate were not taken into account. At each study, the means obtained for each replicate (N = 3) were analysed. An analysis of variance (F test) determined whether the means differed significantly. When this was the case, a two-by-two means comparison test was performed (*t*-test, maximum risk 2.5%).

ANALYSIS OF **RIA AND EIA** RESULTS. Concentrations of PGE_2 and 6-keto $PGF_{1\alpha}$ were determined by immunoassay. Concentrations were determined by interpolation of sample responses on a standard smoothed curve. Concentrations were corrected by dilution factor and extraction yield, then normalised to the control. Only results situated in a reading zone defined by detection limits (dilution giving a B/B_0 response between 10 and 90%) were included. For each sample, the mean and standard deviation were calculated on all interpretable values after rejection of values giving a coefficient of variation greater than 15% (a minimum of two items of data had to be included in the calculation).

In Vivo *Studies (Oedema of the paw)*

Groups of Wistar Rats (Charles River WI BR) of both sexes were used in the studies (no differences were observed in the response of males and females): mean weight 120 to 140 g, fasted for 24 hr, and with free access to drinking water. Oedema was induced by plantar injection of 0.1 mL of physiological saline with 1 mM Ca^{2+} added and containing either 0.1 μg of soluble exogenous PLA_2 (*Naja mossambica mossambica* = 0.15 U of PLA_2) or 0.01 μg of melittin (indicating the presence of less than 2×10^{-4} U of PLA_2). Paw volume was measured by means of a mercury plethysmograph 30 min, 1 hr, and 2 hr after injection of the inflammation-inducing agent. Variations in plantar volume were expressed in mL. Activity was expressed as percentage inhibition of plantar volume measured in each of the groups treated and for each timepoint, compared with plantar volume in the control group studied under the same experimental conditions. Each point represents the study of between 8 and 24 rats. Statistical analysis was performed on individual values by means of the Student's *t*-test.

RESULTS

In Vitro *Results*

PRESENCE OF **cPLA$_2$, sPLA$_2$, COX-1,** AND **COX-2** IN **CRL1517** CELLS. Figure 1 describes all the enzymes being present within the cells before and after stimulation by mellitin. COX-2 has also been reported to be present in unstimulated HUVEC [11].

STIMULATION OF **PLA_2** ACTIVITY BY MELITTIN, BRADYKININ AND IONOMYCIN. Figure 2 shows a significant increase in the release of labelled arachidonic acid from HUVEC, which was dose-dependent between 0.14 and 0.42 μM melittin. Higher concentrations (above 0.7 μM) of melittin were found to be toxic. The concentration of 0.42 μM melittin was therefore used for the following experiments. Similarly, 0.42 μM melittin, 1 μM bradykinin, and 1 μM ionomycin were shown to increase the release of labelled arachidonic acid from CRL1517 cells (not shown).

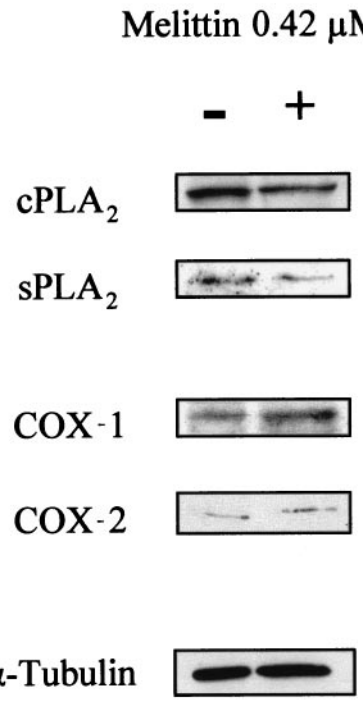

FIG. 1. Western blot of PLA_2s and COXs in CRL1517 with or without stimulation by melittin. Cells were either stimulated or not with 0.42 μM melittin for 30 min. Membranes were immunostained with either anti- $sPLA_2$, -$cPLA_2$, -COX-1, or -COX-2 antibodies and with α tubulin as an internal standard.

EFFECTS OF ALMINOPROFEN, DEXAMETHASONE, INDOMETHACIN, FLURBIPROFEN AND AACOCF3 ON *IN VITRO* PLA_2 ACTIVITY. HUVEC prepared with [^{3}H]arachidonic acid were incubated with alminoprofen (10 μM), quinacrine (10 μM) or dexamethasone (1 μM), and then stimulated by melittin. Figure 3A shows that the three substances induced a significant inhibition of arachidonic acid release of 34, 41, and 57%, respectively.

Dose–response curves were performed on the CRL 1517 cell line with alminoprofen, using three different stimuli: 0.42 μM melittin, 1 μM bradykinin, and 1 μM ionomycin. Figure 3B shows that, whatever the simulus used, alminoprofen induced a similar dose-related inhibition plateauing at 50% inhibition.

Dose–response curves were performed with indomethacin, flurbiprofen, AACOCF3, and dexamethasone after stimulation by 0.42 μM melittin. As reported in Fig. 3C, AACOCF3 and dexamethasone both induced a dose-related inhibition of PLA_2 activity, whereas indomethacin and flurbiprofen were devoid of any inhibitory effect in the same concentration range as alminoprofen.

EFFECTS OF ALMINOPROFEN TOGETHER WITH INDOMETHACIN, FLURBIPROFEN, AND L745337 ON PROSTAGLANDIN RELEASE *IN VITRO*. Prostaglandins were assayed under the same experimental conditions as before in the supernatants of cell cultures which had been used for determination of PLA_2 activity. In HUVEC, the amounts of 6-keto $PGF_{1\alpha}$, the metabolite of prostacyclin, were always higher than those of PGE_2. As reported, in endothelial cells [11], prostacyclin is the principal metabolite of arachidonic acid. This metabolite was not measured in the fibroblasts.

Production of PGE_2 and of 6-keto $PGF_{1\alpha}$ was very low under basal conditions. Stimulation of cells by melittin induced a clear and dose-dependent increase in the concentrations of these two metabolites (not shown). Under our experimental conditions, 10 μM alminoprofen and quinacrine, and 1 μM dexamethasone induced an inhibition of PGE_2 production of 49, 60, and 58%, respectively and an inhibition of 6-keto $PGF_{1\alpha}$ production of 53, 57, and 58%, respectively (Fig. 4A). Alminoprofen (from 0 to 50 μM) induced a dose-related inhibitory response on the levels of PGE_2 formed by the CRL-1517 cell line (not shown). The specific COX-2 inhibitor L745 337 (from 0 to 100 nM) also induced a dose-related inhibitory response on the levels of PGE_2 (Fig. 4B).

These experiments were designed to choose a concentration of alminoprofen (10 μM) that gave an incomplete reduction in the PGE_2 levels and analyse whether other COX inhibitors at concentrations under their IC_{50}s could increase the inhibitory effect [4]. When using such a concentration of the mostly COX-1 inhibitors indomethacin or flurbiprofen (0.1 μM), the inhibition of the stimu-

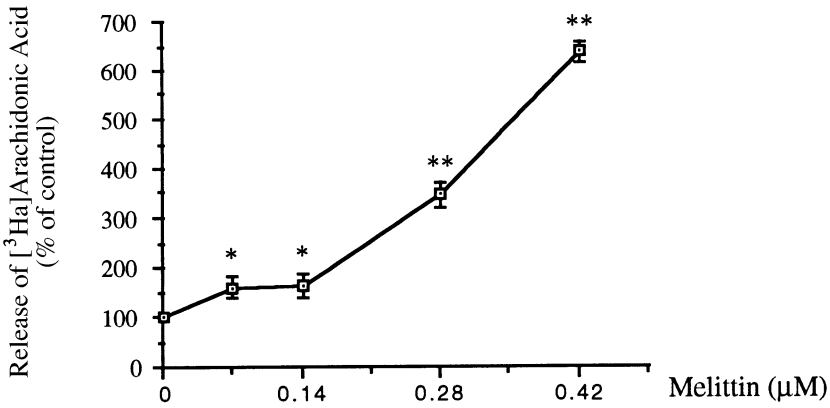

FIG. 2. [^{3}H]Arachidonic acid release from prelabelled HUVEC in response to melittin. HUVEC were stimulated for 30 min with increasing concentrations of melittin. Results are expressed as the release of [^{3}H] arachidonic acid in treated versus control cells and are given in percentage of control. *$P < 0.025$, ** $P < 0.001$.

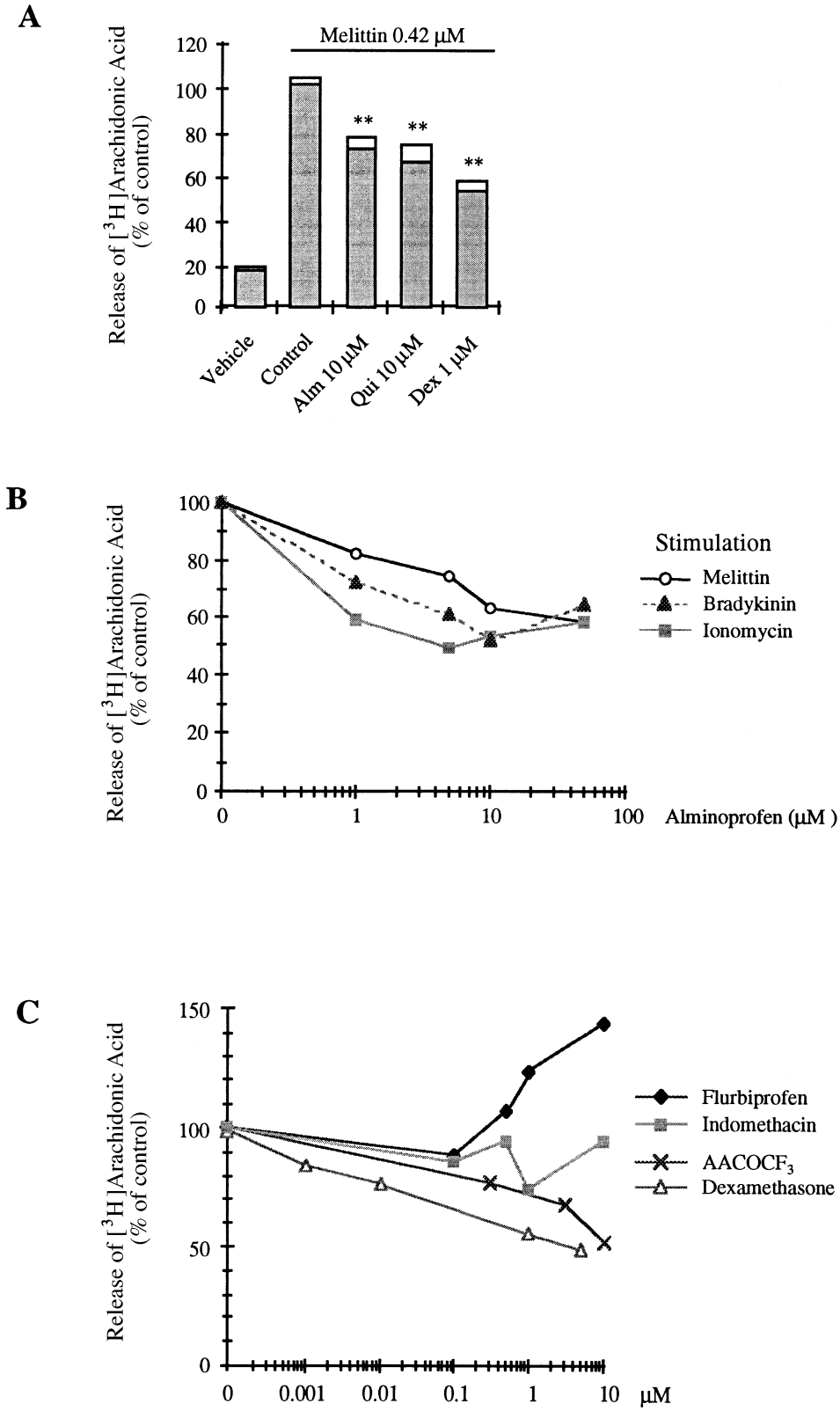

FIG. 3. Effects of alminoprofen and other anti-inflammatory drugs on [^{3}H] arachidonic acid release after melittin stimulation. Cells were treated 20 for min with anti-inflammatory drugs, then for a further 30 min with melittin (0.42 μM). Results are expressed as [^{3}H] arachidonic acid released in treated cells versus melittin-stimulated cells taken as the control (100%). (A) HUVEC: comparative effects of alminoprofen (10 μM), Quinacrine (10 μM), and dexamethasone (1 μM). **$P < 0.001$. The white bars represent the standard deviation values. (B) Fibroblasts CRL 1517: dose-response of alminoprofen (1, 5, 10, and 50 μM) after three different stimulations: 0.42 μM melittin, 1 μM bradykinin, and 1 μM ionomycin. (C) Fibroblasts CRL 1517: dose-response of dexamethasone (1 nM, 10 nM, 1 μM, and 5 μM), AACOCF3 (0.3, 3, and 10 μM), and indomethacin and flurbiprofen (0.1, 0.5, 1, and 10 μM).

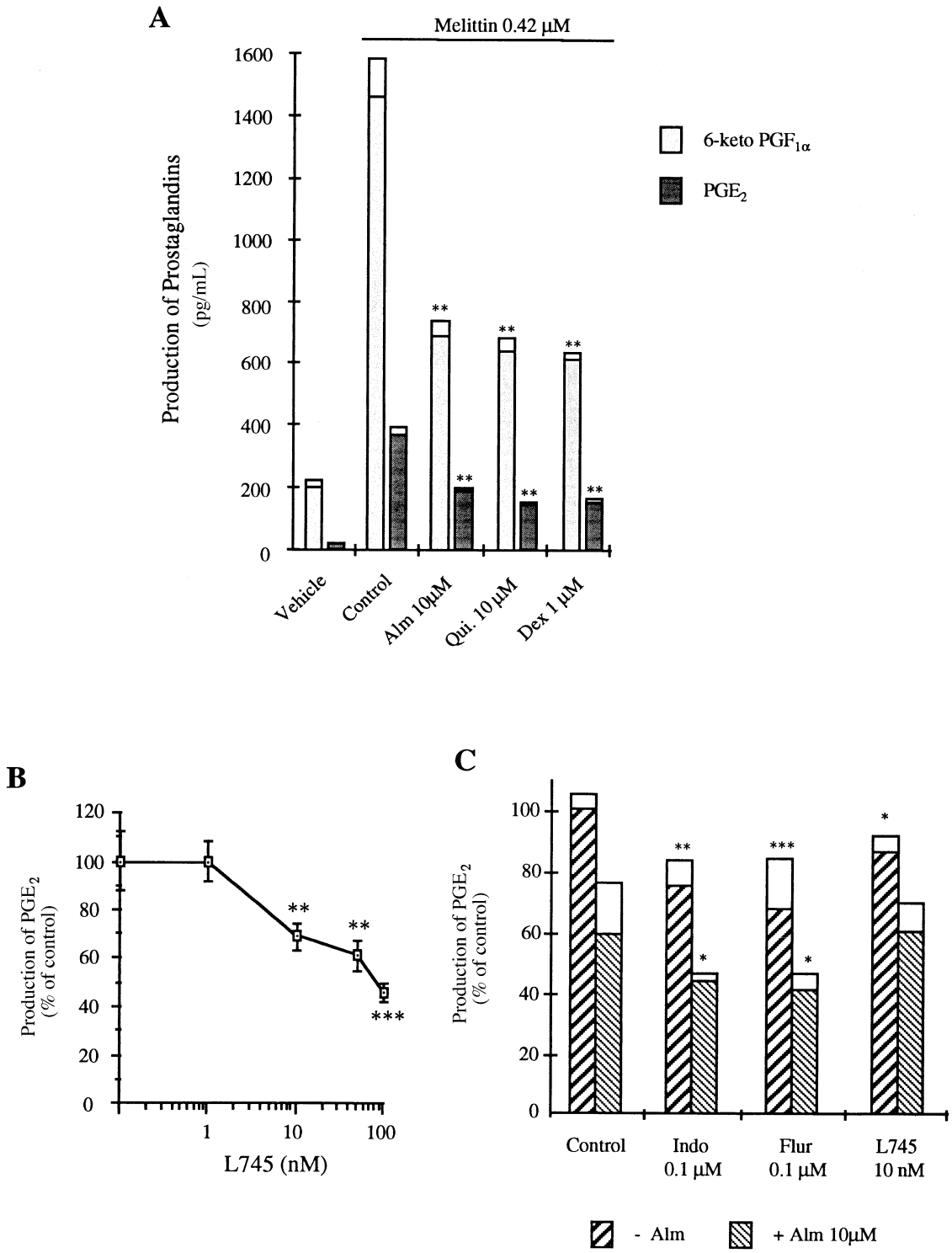

FIG. 4. Effects of alminoprofen and other anti-inflammatory drugs on prostaglandin production after melittin stimulation. Cells were treated for 20 min with anti-inflammatory drugs, then for a further 30 min with melittin (0.42 µM). *$P < 0.05$, **$P < 0.001$, and ***$P < 0.0005$. The white bars represent the standard deviation values. (A) HUVEC: concentrations in pg/mL. Comparative effects of alminoprofen (10 µM), quinacrine (10 µM) and dexamethasone (1 µM) on 6-keto-$PGF_{1\alpha}$ and PGE_2 production. (B) Fibroblasts CRL 1517: results are expressed as PGE_2 produced in L745 337-treated cells versus melittin-stimulated cells taken as the control. Dose-response of L745 337 (1, 10, 50, and 100 nM). (C) Fibroblasts CRL 1517: results are expressed as PGE_2 produced in treated cells versus melittin-stimulated cells taken as the control (100%). Effects on PGE_2 production of alminoprofen (10 µM) with or without indomethacin (0.1 µM), flurbiprofen (0.1 µM) or L745 337 (10 nM). Statistical analyses were performed in control cells (melittin alone) versus indomethacin-, flurbiprofen-, or L745 337-treated cells (left columns) on the one hand and in alminoprofen-treated cells versus indomethacin-, flurbiprofen-, or L745 337- plus alminoprofen-treated cells (right columns) on the other.

latory effect of melittin was 25% for indomethacin and 33% for flurbiprofen. The addition of 10 μM of alminoprofen exerted a significant additive effect on the inhibition (Fig. 4C). The inhibitory effect of 10 nM L745 337 on melittin stimulation was 14%, but the addition of 10 μM alminoprofen to these L745 337-treated cells did not induce any further inhibition as compared to the control cells. As reported by Fujiyoshi [4], alminoprofen at this concentration has no inhibitory effect on COX-1. Taken together, these results could suggest but not prove an effect of alminoprofen on COX-2.

EFFECT OF **DTT** ON **PLA_2** ACTIVITY AND **PGE_2** LEVELS *IN VITRO*. Treatment of HUVEC previously labelled with arachidonic acid with increasing doses of DTT induced a dose-dependent inhibition of the melittin-stimulated PLA_2 activity (not shown). At a concentration of 5 mM DTT, inhibition reached 50% and did not have any effect on cell viability (not shown). Alminoprofen (1, 5, and 10 μM) and 10 μM quinacrine did not increase the inhibition induced by DTT. Similarly, a concentration of 1 μM dexamethasone did not cause any potentiation of PLA_2 activity inhibition (Fig. 5A).

Similar experiments were performed in the CRL-1517 cell line, using 1 μM indomethacin, 1 μM flurbiprofen, 10 μM alminoprofen and 1 μM dexamethasone together with 5 mM DTT. Both PLA_2 activity and PGE_2 production were measured. As found in HUVEC, alminoprofen and dexamethasone did not induce any further PLA_2 inhibition (Fig. 5B). As expected, neither indomethacin nor flurbiprofen had any effect on PLA_2 under these conditions. On the contrary, PGE_2 levels were further inhibited by all drugs, indicating an inhibition of a COX (Fig. 5C). Taken together, these results suggest that alminoprofen is also a COX inhibitor.

Since our data suggest that alminoprofen may inhibit $sPLA_2$ and not $cPLA_2$, we used together concentrations of alminoprofen and AACOCF3 under their IC_{50}s and analysed their inhibitory effect on the release of labelled arachidonic acid in CRL 1517 cells stimulated by 0.42 μM melittin. AACOCF3 (0.3 μM) gave 23% ± 0.29 (mean ± SEM, N = 6) inhibition, alminoprofen (10 μM) gave 26% ± 0.47 (mean ± SEM, N = 6) inhibition and when added together, AACOCF3 and alminoprofen induced a 32% ± 0.26 (mean ± SEM, N = 6) inhibitory effect. Taken together, these results suggest that alminoprofen is most likely a sPLA2 inhibitor.

IN VITRO EFFECT OF ALMINOPROFEN ON **COX-1** AND **COX-2.** As reported in Table 1, alminoprofen inhibited COX-2 activity and had no effect on COX-1 activity at the highest dose used in our *in vitro* experiments (50 μM). Indomethacin inhibited COX-1 at 5 μM while L 745 337 inhibited COX-2 at 1 μM.

In Vivo *Results*

EFFECTS OF ALMINOPROFEN ON PLANTAR OEDEMA INDUCED IN THE RAT BY EXOGENOUS **PLA_2.** All substances, with the exception of dexamethasone, were given orally 1 hr before plantar injection in suspension in 5% gum arabic in a volume of 1 mL/100 g of bodyweight. Dexamethasone in the form of metasulphobenzoate was given subcutaneously in water in a volume of 0.2 mL/100 g of body weight 3 hr before. The doses used represent a range situated between the doses currently used in the rat for their anti-inflammatory activity in classical tests and the unit dose used in man (dose per tablet). Calcium was provided at a millimolar concentration necessary for activity of venom-type secreted PLA_2 [12, 13].

Plantar injection of exogenous PLA_2 (*Naja mossambica mossambica*) provoked an inflammatory response which appeared rapidly during the first 30 min. This reaction was intense, painful, and was accompanied by major vascular phenomena (Table 2A). In this model, alminoprofen from 100 $mg.kg^{-1}$ exerted a statistically significant dose-dependent antagonist action lasting throughout the 2-hr observation period (Table 2A). The maximum change, which occurred 30 min after injection of PLA_2, was 21.2, 27.2, and 50.3%, respectively for 100, 200, and 300 $mg.kg^{-1}$ of substance.

Under the same experimental conditions, only ASA, of the NSAIDs studied (ibuprofen, ketoprofen, diclofenac, tiaprofenic acid, piroxicam, indomethacin, and ASA), exerted a statistically significant action, with maximum inhibition of 18.9% at a dose of 500 $mg.kg^{-1}$ (Table 2B). In contrast, dexamethasone, which is known to have activity against PLA_2, showed more marked efficacy: inhibition of 38.3% was recorded for 1 $mg.kg^{-1}$ of substance, 30 min after injection of the inflammation-inducing agent. This was also true for quinacrine (a nonspecific PLA_2 inhibitor) and for phenidone (a dual inhibitor), which induced a marked and statistically significant decrease in plantar volume.

ACTION OF ALMINOPROFEN ON PLANTAR OEDEMA INDUCED IN THE RAT BY MELITTIN. In this model of melittin-induced oedema, alminoprofen also developed activity which increased in intensity and duration in relation to the dose administered. The efficacy of the substance was low at 20 and 50 $mg.kg^{-1}$ (−11.7 and −14.9%, not shown). In contrast, the efficacy of the substance was greater at higher doses, with maximum inhibition of oedema reaching 24.5, 30.2, and 41.5%, respectively for 100, 200, and 300 $mg.kg^{-1}$ after 30 min; these changes, although decreasing over time, remained statistically significant throughout the study (Table 3A).

As in the previous study, the results recorded with the other tested substances in this experimental oedema model showed that of the arylcarboxylic derivatives, only ibuprofen exerted some minor activity at doses in the range of 200 to 400 $mg.kg^{-1}$; however, this action remained very much lower than that observed with alminoprofen. Piroxicam and indomethacin remained inactive. In contrast, a statistically significant action was recorded for the other three

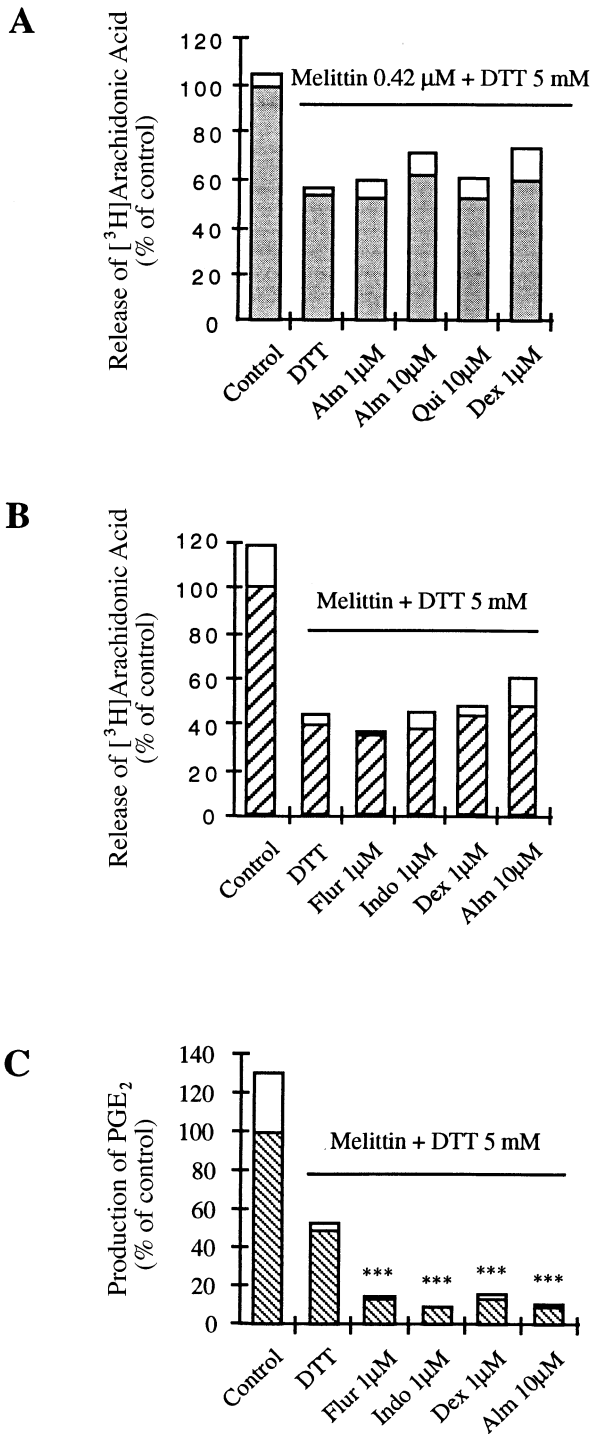

FIG. 5. Influence of DTT on drug efficiency after melittin stimulation. Cells were incubated with or without (control) 5 mM DTT, then treated as previously described. Results are expressed as [^{3}H] arachidonic acid or PGE_2 produced in treated cells versus melittin-stimulated cells taken as the control. Statistics are given comparing melittin plus DTT-stimulated cells with those receiving the other drugs, ***$P < 0.0005$. The white bars represent the standard deviation values. (A) HUVEC: influence of DTT on inhibition of [^{3}H] arachidonic acid release by alminoprofen (1 or 10 μM), quinacrine (10 μM), and dexamethasone (1 μM). (B) Fibroblasts CRL 1517: influence of DTT on inhibition of [^{3}H]arachidonic acid release by flurbiprofen (1 μM), indomethacin (1 μM), and dexamethasone (1 μM) and alminoprofen (10 μM). (C) Fibroblasts CRL 1517: influence of DTT on inhibition of PGE_2 production by flurbiprofen (1 μM), indomethacin (1 μM), and dexamethasone (1 μM) and alminoprofen (10 μM).

TABLE 1. Inhibition of COX activities measured *in vitro*

Enzyme	Treatment	% Inhibition
COX 1	Indomethacin 5 μM	33% ± 6.58 (N = 6)†
	Alminoprofen 50 μM	3% ± 0.77 N = 3)
COX 2	L745 337 1 μM	24% ± 8.78 (N = 4)
	Alminoprofen 50 μM	34% ± 6.35 (N = 4)*

Enzymatic reactions using either COX-1 or COX-2 were performed *in vitro* at 37° using arachidonic acid as a substrate. PGE_2 were measured by EIA.
* $P < 0.05$, † $P < 0.005$.

classes of substances, represented by quinacrine, phenidone, and dexamethasone, although the activity of the latter was more transient (Table 3B).

DISCUSSION

Our results clearly demonstrate that alminoprofen, both *in vitro* and *in vivo*, mimicks the action of dexamethasone, an anti-inflammatory steroid, more than the action of other NSAIDs. Indeed, in the *in vitro* model of human cells, alminoprofen inhibits the release of incorporated tritiated arachidonic acid induced by melittin, a powerful activator of PLA_2 as well as the formation of prostaglandins to the same extent as quinacrine and dexamethasone, both known inhibitors of PLA_2. In the *in vivo* model of rat paw oedema induced either by venom PLA_2 or melittin, alminoprofen exerts an anti-inflammatory activity similar to that of dexamethasone, quinacrine, and phenidone, whereas classical NSAIDs have no effect. It should be noted that our results are not in agreement with those of Hartman *et al.* [14] in a mouse paw oedema model, where they reported that the effects of melittin were not inhibited by PLA_2 inhibitors (scaladarial and dexamethasone) but by antihistaminic compounds. We propose that this discrepancy may be due to the fact that under our experimental conditions, we used 1000 times less melittin than these authors (10 ng versus 10 μg). This may indicate that at low concentrations, melittin mainly activates the PLA_2, whereas at much higher concentrations, it may elicit a different sequence of inflammatory events, mostly histamine release. In any case, our results support the initial hypothesis advanced by

TABLE 2. Oedema induced by PLA_2 in the presence of 1 mM calcium

Product	Dose ($mg.kg^{-1}$)	30 min	1 hr	2 hr
A				
Control*		.63 mL	.78 mL	.83 mL
Alminoprofen	100	−21.2%†	−18.2%†	−10.6%‡
	200	−27.2%†	−27.8%†	−16.6%§
	300	−50.3%†	−42.2%†	−24.6%†
B				
Ibuprofen	300	+5.3%	+5.7%	0%
Ketoprofen	50	+6.7%	+6.8%	+4.2%
Diclofenac	50	+13.2%‡	+4.7%	−1.4%
Tiaprofenic acid	100	−5.7%	−6.3%	+2.8%
Acetylsalicylic acid	500	−18.9%§	−18.8%†	−4.2%
Piroxicam	20	−12.1%	−7.0%	−5.0%
Indomethacin	25	+5.7%	+14.3%	+12.7%
Quinacrine	200	−74.1%†	−66.7%†	−57.0%†
Phenidone	100	−63.8%†	−56.3%†	−50.0%†
Dexamethasone	1	−38.3%§	−22.6%	−15.0%

Percentage of plantar volume (%). Oedema is induced by injection of 0.1 μg exogenous PLA_2 in physiological solution containing 1 mM Ca^{2+}. Drugs are given orally 1 hr before injection, except dexamethasone given subcutaneously 3 hr before.

(A) Control and effects of alminoprofen.

(B) Effects of other anti-inflammatory drugs.

* Control is expressed in variation of plantar volume (mL).

†$P < 0.001$. ‡$P < 0.05$. §$P < 0.01$.

Fujiyoshi, i.e. that the anti-inflammatory activity of alminoprofen cannot be explained only by its relatively weak anticyclooxygenase properties or by its absence of antihistaminic properties [4]. The mechanism of action of alminoprofen must therefore involve another point of attack on the arachidonic acid cascade.

Since dexamethasone is known to inhibit PLA_2 activity and quinacrine is known as a nonspecific inhibitor of PLA_2, we propose that part of the anti-inflammatory effect of alminoprofen is due to its ability to inhibit a PLA_2 involved in the release of arachidonic acid after melittin stimulation.

TABLE 3. Oedema induced by melittin in the presence of 1 mM calcium

Product	Dose ($mg.kg^{-1}$)	30 min	1 hr	2 hr
A				
Control*		.66 mL	.75 mL	.83 mL
Alminoprofen	100	−24.5%†	−20.0%†	−16.7%§
	200	−30.2%‡	−26.7%‡	−21.2%†
	300	−41.5%‡	−33.3%‡	−25.8%‡
B				
Ibuprofen	300	−17.5%§	−16.4%†	−10.4%
Ketoprofen	50	+22.2%§	+11.5%	+4.1%
Diclofenac	50	+3.4%	+1.4%	−5.9%
Tiaprofenic acid	100	+3.4%	−1.4%	−4.7%
Piroxicam	20	+4.1%	−1.6%	−9.0%
Indomethacin	25	−3.4%	−2.8%	−4.7%
Quinacrine	200	−55.6%‡	−53.7%‡	−50.0%‡
Phenidone	100	−39.2%‡	−33.8%‡	−23.9%†
Dexamethasone	1	−30.6%§	−14.1%	−10.3%

Percentage of plantar volume (%). Oedema is induced by injection of 10 ng of melittin in physiological solution containing 1 mM Ca^{2+}. Drugs are given orally 1 hr before injection, except dexamethasone given subcutaneously 3 hr before.

(A) Control and effects of alminoprofen.

(B) Effects of other anti-inflammatory drugs.

* Control is expressed in variation of plantar volume (mL).

†$P < 0.01$. ‡$P < 0.001$. §$P < 0.05$.

At the present time, two types of PLA_2 have been implicated at least in the release of arachidonic acid: a cytosolic PLA_2 involved in the signalling of many inflammatory mediator receptors such as interleukin-1, tumor necrosis factor, interleukin-8 and other chemokines and chemoattractants, and a secreted PLA_2 [12, 13]. This PLA_2 of 14 kDa is secreted by inflammatory cells [15], is found in a great variety of inflammatory tissues [16, 17], and is involved in most inflammatory reactions [18–20]. We wondered, therefore, whether alminoprofen inhibited the $cPLA_2$ or the $sPLA_2$. Using the cultured HUVEC, we looked at the effect of DTT. It is known that cytosolic PLA_2 does not have disulphide bridges, while secreted PLA_2 requires intact disulphide bridges for its activity. Therefore, DTT will inhibit the action of the secreted PLA_2 by inhibiting the formation of the disulphide bridges. In our experimental model, the combination of alminoprofen with DTT did not increase inhibition of the PLA_2 activity at any of the doses studied. This apparent loss of anti-PLA_2 activity by alminoprofen suggests that the substance has an inhibitory effect on secreted PLA_2 as do quinacrine and dexamethasone.

Although our results strongly support the hypothesis of an effect of alminoprofen on PLA_2, some of our data indicate that alminoprofen may also have an effect on a COX. Indeed, after treating the cells with DTT, which inhibits the secretory PLA_2, the addition of alminoprofen as well as the other NSAIDs and dexamethasone inhibit PGE_2 formation even further. These observations indicate that alminoprofen exerts an inhibitory action on one of the COX isoforms. The suggestion that the COX-2 isoform may be the target of alminoprofen comes firstly, from the data reported by Fujiyoshi [4] showing that alminoprofen did not inhibit the COX found in sheep seminal vesicle microsomal fraction (namely COX-1); secondly, from the experiments described in Fig. 4C indeed, when adding low doses of NSAIDs (preferentially either COX-1 inhibitors such as indomethacin and flurbiprofen or a COX-2 inhibitor such as L-745 337) together with alminoprofen and measuring the production of PGE_2 after melittin stimulation, we only find an additive effect on the inhibition of PGE_2 production with COX-1 inhibitors, whereas this does not occur with the COX-2 inhibitor; and thirdly, from our *in vitro* experiments using purified COX-1 and COX-2 which demonstrate an inhibitory effect of alminoprofen on COX-2 but not on COX-1 at the doses used to inhibit PG production. Taken together, these results suggest that alminoprofen must preferentially interact with COX-2.

The mechanism of action of alminoprofen towards both secreted PLA_2 and COX-2 will require further investigation. However, this dual action could lead to the development of anti-inflammatory drugs that would have a good anti-inflammatory effect without the side-effects of COX-1 inhibitors and corticosteroids.

References

1. Fujiyoshi T, Iida H and Hayashi I, Pharmacological profile and mode of action of alminoprofen, a non steroidal anti inflammatory drug. *Jap Pharmacol Ther* **18:** 159–180, 1990.
2. Pruzanski W and Vadas P, Phospholipase A_2 a mediator between proximal and distal effectors of inflammation. *Immunol Today* **12**(5): 143–146, 1991.
3. Vane JR and Botting RM, New insights into the mode of action of anti-inflammatory drugs. *Inflamm Res* **44:** 1–10, 1995.
4. Fujiyoshi T, Iida H, Murakami M, Kuwashima M and Uematsu T, Pharmacological studies on EB 382, a new non steroidal anti inflammatory agent: mode of action of the analgesic effects. *Chem Pharm Bull* **37:** 775–777, 1989.
5. Cirino G, Peers SH, Wallace JL and Flower RJ, A study of phospholipase A_2-induced oedema in rat paw. *Eur J Pharmacol* **166:** 505–510, 1989.
6. Shikada K and Tanaka S, Influence of epithelium on the inhibition of melittin-induced contraction of guinea-pig isolated trachea by the potassium channel opener NIP-121. *Br J Pharmacol* **109:** 1091–1096, 1993.
7. Camorato AM and Grandison L, Evidence supporting a correlation between arachidonic acid release and prolactin secretion from CH_3 cells. *Endocrinology* **116:** 1506–1513, 1983.
8. Vicart P, Testut P, Schwartz B, Liorens-Cortes C, Perdomo JJ and Paulin D, Cell adhesion markers are expressed by a stable human endothelial cell line transformed by the SV40 large T antigen under vimentin promoter control. *J Cell Physiol* **157:** 41–51, 1993.
9. Comera C, Rothhut B and Russo-Marie F, Identification and characterization of phospholipase A_2 inhibitory proteins in human mononuclear cells. *Eur J Biochem* **188:** 139–146, 1990.
10. Dray F, Vuilliez-Le Normand B, Deroussent A, Briquet I, Gabellec MM, Nakamura S, Wahl LM, Gouyette A and Salahuddin SZ, Active metabolism of arachidonic acid by Kaposi sarcoma cells cultured from lung biopsies; identification by HPLC and MS/MS of the predominant metabolite secreted as the 11.12-epoxy-eicosatrienoic acid. *Biochim Biophys Acta* **1180:** 83–90, 1992.
11. Karim S, Habib A, Levy-Toledano S and Maclouf J, Cyclooxygenases-1 and -2 of endothelial cells utilize exogenous or endogenous arachidonic acid for transcellular production of thromboxane. *J Biol Chem* **271:** 12042–12048, 1996.
12. Bonventre J, Phospholipase A_2 and signal transduction. *J Am Soc Nephrol* **3:** 128–150, 1992.
13. Glaser KB, Mobilio D, Chang JY and Senko N, Phospholipase A_2 enzymes: Regulation and inhibition. *Tips* **14:** 92–98, 1993.
14. Hartman DA, Tomchek LA, Lugay JR, Lewin AC, Chau TT and Carlson RP, Comparison of the anti-inflammatory and anti-allergic drugs in the melittin- and D49 PLA2-induced mouse paw oedema models. *Agents Actions* **34:** 84–88, 1991.
15. Vadas P and Pruzanski W, Biology of disease: role of secretory phospholipase A_2 in the pathobiology of disease. *Lab Invest* **55:** 391–404, 1986.
16. Forster S, Ilderton E, Norris JFB, Summerly R and Yardly HJ, Characterisation and activity of phospholipase A_2 in normal human epidermis and in lesion-free epidermis of patients with psoriasis or eczema. *Br J Dermatol* **112:** 135–141, 1985.
17. Vadas P, Stefanski E and Pruzanski W, Characterisation of extracellular phospholipase A_2 in rheumatoid synovial fluid. *Life Sci* **36:** 579–584, 1985.
18. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA and

Serhan CN, Leukotrienes and lipoxins: structures, biosynthesis and biological effects. *Science* **237:** 1171, 1987.
19. Zipser RD and Giacomo L, Prostaglandins, thromboxanes and leukotrienes in clinical medicine. *Western J Med* **143:** 485–489, 1985.
20. Fourcade O, Simon MF, Viodé C, Rugani N, Leballe F, Ragab A, Fournié B, Sarda L and Chap H, Secretory phospholipase A$_2$ generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* **80:** 919–927, 1995.