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Suppression of cytokine synthesis, integrin expression and chronic inflammation by inhibitors of cytosolic phospholipase A_2

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Abstract

To define the isoform of phospholipases A_2 active in inflammation we evaluated the effects of low-molecular-weight inhibitors of secretory and cytosolic phospholipases A_2. We found that inhibitors of cytosolic phospholipase A_2 had therapeutic efficacy in an in vivo model of chronic inflammation (rat adjuvant arthritis), whereas inhibitors of secretory phospholipase A_2 had no beneficial effect. In vitro, inhibitors of cytosolic phospholipase A_2 diminished surface expression of Mac-1 (CD11b/CD18) β_2-integrin on calcium ionophore-stimulated human blood granulocytes and suppressed synthesis of interleukin-1β in lipopolysaccharide-stimulated human blood monocytes and U937 cells by reducing mRNA levels. Lipid mediators promote Mac-1 exocytosis and transcription of interleukin-1β, which further enhances cytosolic phospholipase A_2 activity and expression. Thus, superinduction of cytosolic phospholipase A_2 may establish a positive feedback loop, converting acute inflammation into chronic inflammation. Consequently, inhibitors of cytosolic phospholipase A_2 may prevent inflammation in vivo by interfering with cellular activation and infiltration. We conclude that cytosolic phospholipase A_2 but not secretory phospholipase A_2 is the predominant enzyme in inflammatory signalling.

Keywords: Phospholipase A_2; Inflammation; Phospholipase inhibitor; Interleukin-1; Integrin

1. Introduction

Signal-mediated activation of phospholipases A_2 is thought to be a prime event in inflammation, resulting in numerous acute and chronic disease states (Kaiser et al., 1990; Vadas et al., 1993). Phospholipases A_2 contribute to the pathogenesis by attacking cellular membranes, releasing proinflammatory lipid mediators. Cleavage of phospholipids yields free arachidonic acid, which is further metabolized into prostaglandins and leukotrienes, and lysophospholipids, which are a source for the synthesis of platelet activating factor. Besides their role in paracrine signalling leading to bronchoconstriction, chemotaxis and increased vasopermeability, these mediators promote exocytosis and regulate a variety of enzymes involved in signal transduction by acting as intracellular 'second messengers' (Exton, 1994). Additionally, eicosanoids and platelet activating factor stimulate the expression of genes for adhesion molecules (integrins) (Milam et al., 1991), proinflammatory cytokines (interleukin-1β, tumor necrosis factor-α, interleukin-6) (Rola-Pleszczynski and Lemaire, 1985; Barthelson and Valone, 1991; Poubelle et al., 1991; Brach et al., 1992) and tissue proteases (collagenases) (Bazan et al., 1993). In mammals two isoforms of phospholipases A_2, a 14-kDa secretory and a 85-kDa cytosolic phospholipase A_2, have been identified, which do not share sequence homology and differ in their catalytic mechanism and regulation (Dennis, 1994; Winkler et al., 1994). The cytosolic phospholipase A_2 is located in the cytosol of quiescent cells, e.g., platelets and leukocytes (Marshall and Roshak, 1993; Rodewald et al., 1994). Activation of cells by lipopolysaccharide (Mohri et al., 1990; Doerfler et al., 1994), thrombin (Kramer et al., 1993) or cytokines (interleukin-1β, tumor necrosis factor-α) (Hoeck et al., 1993; Angel et al., 1994) leads to rapid phosphorylation of the enzyme by protein kinase C (Wijkander and Sundler,

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1992) and mitogen-activated protein kinase (Lin et al., 1993), followed by translocation to the plasma membrane, where it binds to phospholipid substrate depending on micromolar Ca^{2+} concentrations. Moreover, sustained de novo synthesis of cytosolic phospholipase A_2 (Hoeck et al., 1993; Rodewald et al., 1994) is observed after stimulation with cytokines and lipopolysaccharide. The cytosolic phospholipase A_2 shows preference for phospholipid substrates esterified with arachidonic acid in position *sn-2* of glycerol, whereas the secretory phospholipase A_2 exhibits no selectivity for fatty acids (Dennis, 1994; Winkler et al., 1994). Accordingly, it is assumed that cytosolic phospholipase A_2 is the enzyme responsible for intracellular signal-mediated release of arachidonic acid. In contrast, secretory phospholipase A_2 is preformed in granules (Rosenthal et al., 1995) of platelets, mast cells, synoviocytes or hepatocytes and is exocytosed after activation of cells by cytokines (interleukin-1β, tumor necrosis factor-α, interleukin-6) or lipopolysaccharide (Vadas et al., 1993). Additionally, de novo synthesis of secretory phospholipase A_2 can be induced by these agents as it is in an acute-phase response (Crowl et al., 1991; Vadas et al., 1993). The secretory phospholipase A_2 is assumed to be active only in the extracellular space in the presence of millimolar concentrations of Ca^{2+} and is responsible for degradation and membrane remodeling in inflamed or injured tissue (Winkler et al., 1994). Accordingly, elevated levels of secretory phospholipase A_2 enzyme are detectable in the serum of patients with septic shock or acute pancreatitis and in inflammatory exudates, e.g., synovial fluid of rheumatic joints. High activity of this enzyme is further associated with asthma, psoriasis and arthritis (Kaiser et al., 1990; Vadas et al., 1993).

Since data aiming to correlate cytosolic phospholipase A_2 activity with pathological states of inflammatory diseases are not available yet, controversy about the role of the two phospholipase A_2 isoforms in inflammation continues. The question whether elevated secretory phospholipase A_2 activity defines the underlying pathogenic mechanism or represents the consequence of dysregulated signalling involving superinduction of cytosolic phospholipase A_2 remains to be answered. Referring to this, our studies investigate the different roles of secretory phospholipase A_2 and cytosolic phospholipase A_2 in inflammation. By using isoform-selective low-molecular-weight inhibitors of secretory and cytosolic phospholipase A_2 activity, we evaluated the impact of the isoforms on the inflammatory response in vitro and in vivo.

Distinct cellular events mark the initiation of inflammation, starting with attachment of granulocytes to endothelial cells mediated by adhesion molecules (selectins, integrins), followed by extravasation into the surrounding tissue. The β_2-integrin Mac-1 (CD11b/CD18), the ligand for ICAM-1 (intercellular cell adhesion molecule-1) expressed on endothelial cells, is preformed in the membrane of peroxidase-negative granules of neutrophils or monocytes and is exocytosed a few minutes after stimulation of cells with platelet activating factor and leukotriene B_4 (Miller et al., 1987; Tonnensen et al., 1989). Since phospholipase A_2 activity is known to be associated with degranulation and attachment of human granulocytes and monocytes (O'Flaherty et al., 1983; Yokote et al., 1993), we probed the effect of isoform specific phospholipase A_2 inhibitors on this 'early' inflammatory event. Completing the scenario, we further investigated the effect of inhibitors of cytosolic and secretory phospholipase A_2 on cytokine synthesis as a 'late' event of the inflammatory response. Transcription of cytokine genes, in particular interleukin-1β (Rola-Pleszczynski and Lemaire, 1985; Barthelson and Valone, 1991; Poubelle et al., 1991), tumor necrosis factor-α (Poubelle et al., 1991) and interleukin-6 (Brach et al., 1992), is enhanced by eicosanoids and platelet activating factor through induction of NF-κB (nuclear factor kappa B), NF-IL6 (nuclear factor interleukin-6) (Brach et al., 1992) and AP-1 (activation protein-1) (Squinto et al., 1989; Danesch et al., 1994). Since these cytokines themselves further enhance the activity and expression of phospholipase A_2 enzymes and since mediators resulting from phospholipase A_2 action induce the expression of cytokines, we and others propose that there is a positive feedback loop (Tibes et al., 1993; Wijkander et al., 1995) leading to phospholipase A_2 superinduction. Consequently, interference with this vicious circle by phospholipase A_2 inhibitors may provide a new pharmacological target for the treatment of inflammatory diseases (Glaser, 1995). Finally, we used a relevant animal model of chronic inflammation (rat adjuvant arthritis) to evaluate the antiphlogistic effect of phospholipase A_2 inhibitors in vivo.

2. Materials and methods

2.1. Phospholipase A_2 inhibition assay

The activity of secretory phospholipase A_2 (human recombinant, Boehringer Mannheim) was evaluated using the test combination 'Free Fatty Acids' (Boehringer Mannheim) as described by Hoffmann and Neumann (1989). Acetylsalicylate, diclofenac and dexamethasone were purchased from Sigma. Compounds labeled with BM numbers (for chemical names and structures see Fig. 1) were synthesized by Boehringer Mannheim including tenidap. Compounds were dissolved in DMSO (dimethyl sulfoxide) at a stock concentration of 10 mg/ml and stored at −20°C. Ten microliters of inhibitor (final concentration: 0.1, 1 and 10 μg/ml, diluted in test buffer) was preincubated with 20 μl of human recombinant secretory phospholipase A_2 (final concentration 18.5 nM, diluted in test buffer) in 96-well plates (F Maxi Sorb, Nunc) for 15 min at 25°C. Thereafter 20 μl of substrate solution (lecithin emulsion dissolved in test buffer) was added and plates were incubated for 1 h at 37°C. Release of fatty

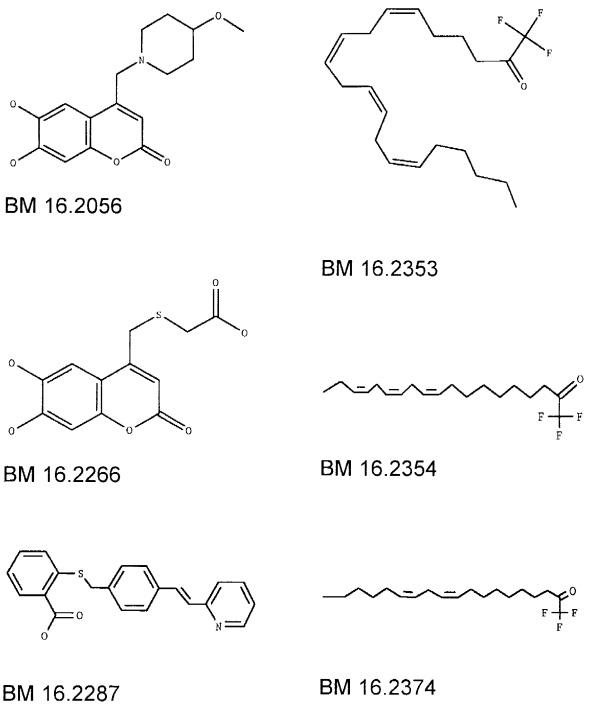

Fig. 1. Chemical structures of compounds used in this study. Inhibitors of secretory phospholipase A_2: BM 16.2056 = 6,7-dihydroxy-4-(4-methoxy-piperidin-1-ylmethyl)-chromen-2-one hydrochloride, molecular weight (m.w.) 341; BM 16.2266 = (6,7-dihydroxy-2-oxo-2*H*-chromen-4-ylmethylsulfanyl)-acetic acid, m.w. 282; BM 16.2287 = 2-(4-(2-pyridin-2-yl-vinyl)-benzylsulfanyl]-benzoic acid sodium salt, m.w. 369. Inhibitors of cytosolic phospholipase A_2: BM 16.2353 = C20:4-$COCF_3$ = 1,1,1-trifluoro-heneicosa-6,9,12,15-tetraen-2-one, m.w. 356; BM 16.2354 = C18:3-$COCF_3$ = 1,1,1-trifluoro-nonadeca-10,13,16-trien-2-one, m.w. 330; BM 16.2374 = C18:2-$COCF_3$ = 1,1,1-trifluoro-nonadeca-10,13-dien-2-one, m.w. 332.

acids by phospholipase A_2 activity was quantified by means of the test combination 'Free Fatty Acids' by measuring the absorbance according to the manufacturer's instructions. The activity of cytosolic phospholipase A_2 was determined with 1-palmitoyl-2-[1-^{14}C]arachidonoyl-L-3-glycerophosphocholine (57 Ci/mol, 50 μCi/ml) as substrate (Amersham). Enzyme activity was determined as described by Rehfeldt et al. (1991) with slight modifications. The phospholipid substrate was diluted 1:9 with toluene/ethanol (1:1, by vol.). This solution was dried under N_2 and suspended in distilled water containing 5 mg/ml bovine serum albumin (essentially fatty acid free, Sigma) by sonication on ice (Labsonic Braun, 50 W, three times for 3 s). In total, 150 μl test buffer (166 mM Tris/HCl, pH 9.5, 8.3 mM $CaCl_2$), 25 μl inhibitor solution (final concentration: 0.1, 1 and 10 μg/ml, diluted in test buffer) and 25 μl of cytosolic phospholipase A_2 (corresponding to 1–5 μg protein) purified from U937 cell line (kindly provided by M. Goppelt-Struebe, University of Erlangen, Germany) were preincubated on ice for 5 min in glass tubes. Subsequently, 50 μl of diluted substrate was added to a final volume of 250 μl. In total, the assay mixture contained 1 μM phospholipid corresponding to approximately 30 000 cpm. After being vortexed tubes were incubated for 30 min under constant shaking (150 rpm) at 37°C. The reaction was terminated by addition of 375 μl cold isopropanol/1 M HCl (1:0.086, by vol.). The glass tubes were kept in an ice bath and 700 μl heptane (Merck) was added. The solution was shaken vigorously and the tubes were kept at 25°C for 15 min to allow the two phases to separate. Thereafter, 500 μl of the heptane phase was transferred to an Eppendorf tube containing 200 μl heptane and approximately 200 mg silica to adsorb extracted phospholipid. After being vortexed tubes were centrifuged at $16\,000 \times g$ for 4 min and 500 μl supernatant was used to determine the radioactivity of released arachidonic acid by scintillation counting.

2.2. Preparation of human peripheral blood subpopulations

Peripheral human venous blood from healthy donors was collected in Liquemin (La Roche, 2500 IU/ml blood) or in 1 mM EDTA (Boehringer Mannheim). Human peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma) according to the manufacturer's instructions. Viability after purification was greater than 98%, as evaluated by exclusion of trypan blue dye (Sigma), yielding an average of 20% monocytes and 80% lymphocytes with some scattered erythrocytes. Platelets were largely removed by several washes with PBS (phosphate-buffered saline, Ca^{2+}/Mg^{2+}-free, Boehringer Mannheim). Mononuclear cells were cultured in RPMI 1640 medium (low endotoxin, Seromed Biochrom) supplemented with 10% fetal calf serum (heat-inactivated for 45 min at 56°C, low endotoxin, Gibco Life Technologies), 2 mM glutamine (Boehringer Mannheim), 1% MEM (minimal essential medium)-vitamins (Gibco Life Technologies), 100 IU/ml penicillin and 20 μg/ml streptomycin (Boehringer Mannheim). Human peripheral blood granulocytes were isolated using Polymorphprep (Nycomed Pharma). Granulocytes were recovered in PBS (Ca^{2+}/Mg^{2+}-free) supplemented with 0.9 mM Ca^{2+}. Viability was greater than 98% and purity was about 80%, with a minor contamination of 20% lymphocytes.

2.3. Cell lines

U937 cells purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM pyruvate (Seromed Biochrom), 1% MEM-nonessential amino acids and 100 IU/ml penicillin and 20 μg/ml streptomycin (Boehringer Mannheim). For differentiation into macrophage-like phenotype (Wiederhold et al., 1988), 0.5×10^6 U937 cells per ml were cultured 3 days in the presence of 50 nM PMA (phorbol 12-myristate 13-acetate) (Sigma). Both adherent

and non-adherent cells were harvested for further experiments. Differentiation was monitored by staining with anti-Mac-1 antibody (CD11b/CD18, mouse IgG1, Boehringer Mannheim), using flow cytometry (see Section 3). The percentage of differentiated, i.e., Mac-1^+, cells ranged from 40% to 60%.

2.4. Detection of Mac-1 by flow cytometry

Freshly isolated peripheral blood granulocytes (2×10^6 per ml) in PBS supplemented with 0.9 mM Ca^{2+} were preincubated with or without inhibitors (at final concentrations of 0.1, 1, 3 and 10 μg/ml) for 15 min at 37°C, 7.5% CO_2 in a humidified atmosphere. The maximum final concentration of the solvent DMSO was 0.1%. Mac-1 exocytosis was initiated with 10 nM Ca^{2+} ionophore A23187 (Boehringer Mannheim) for 10 min at 37°C. Cells were subsequently stained for 30 min with 10 μg/ml FITC (fluorescein isothiocyanate)-labeled Mac-1 antibody (CD11b/CD18, mouse IgG1, Boehringer Mannheim) or with isotype-matched FITC-labeled control antibody (CD22, mouse IgG1, Boehringer Mannheim) in PBS supplemented with 10% fetal calf serum and 0.1% sodium azide. After fixation with 1% *p*-formaldehyde (Sigma) in PBS for 1 h, cells were analyzed on a Becton Dickinson FACScan (fluorescence activated cell scanner) using forward and right angle scatter profiles for granulocyte gating.

2.5. Quantification of cytokine release

Freshly isolated peripheral blood mononuclear cells and PMA differentiated U937 cells (1×10^6 per ml) were incubated in cell culture medium with 100 ng/ml lipopolysaccharide from *Escherichia coli* K235 (Sigma) with or without inhibitors (at final concentrations of 0.1, 1, 3 and 10 μg/ml) at 37°C, 7.5% CO_2 in a humidified atmosphere for 24 h. Thereafter cells were centrifuged at $800 \times g$ for 10 min. Interleukin-1β, tumor necrosis factor-α and interleukin-6 were quantified in the supernatants according to the manufacturer's instructions, using immunoassay kits from Endogen (Biozol).

2.6. Intracellular cytokine detection by flow cytometry

Peripheral blood mononuclear cells and differentiated U937 cells (1×10^6 per ml) were stimulated with 100 ng/ml lipopolysaccharide in the presence or absence of inhibitors (final concentration 10 μg/ml) for the indicated times (see Section 3) in cell culture medium supplemented with 0.5 μM of monensin (Sigma) to prevent secretion of cytokine into the medium (Jung et al., 1993). Cells were fixed in PBS containing 1% *p*-formaldehyde for 10 min. Unspecific binding sites were blocked by incubation of fixed cells for 10 min in RPMI medium supplemented with 10% fetal calf serum and 100 μg/ml goat-IgG (Sigma). Thereafter cells were stained for 1 h with interleukin-1β antibody (mouse IgG1, Boehringer Mannheim) or isotype-matched control antibody (mouse IgG1, Laboserv) in RPMI medium containing 10% fetal calf serum and 0.1% saponin (detergent, Sigma) for permeabilization. Cells were further stained for 30 min with 10 μg/ml FITC-labeled detection antibody (Anti-mouse Ig-fluorescein $F(ab')_2$ fragment, Boehringer Mannheim) in RPMI medium supplemented with 10% fetal calf serum and 0.1% saponin. Free binding sites of FITC-labeled antibody were blocked by incubation of cells for 10 min with 200 μg/ml mouse-IgG (Sigma) in RPMI medium containing 10% fetal calf serum and 0.1% saponin. Additionally, human peripheral blood monocytes were stained with 10 μg/ml phycoerythrin-labeled CD14 antibody (Becton Dickinson) and U937 cells were stained with 10 μg/ml phycoerythrin-labeled Mac-1 (CD11b/CD18) antibody (Becton Dickinson) in RPMI medium with 10% fetal calf serum for 30 min, to monitor differentiation into the macrophage phenotype. Stained cells were subsequently analyzed on a Becton Dickinson FACScan, with fluorescence 1 (FITC) representing intracellular interleukin-1β protein and fluorescence 2 (phycoerythrin) representing Mac-1 or CD14 cell surface bound molecules.

2.7. Cytotoxicity assays

For the propidium iodide cytotoxicity assay, peripheral blood mononuclear cells (1×10^6 per ml) were stimulated in cell culture medium with 100 ng/ml lipopolysaccharide in the presence or absence of inhibitors (final concentrations 0.1, 1 and 10 μg/ml) for 24 h. Monocytes were stained with CD14 antibody (mouse IgM, Boehringer Mannheim) and isotype-matched control antibody (mouse IgM, Biozol) followed by FITC-labeled detection antibody (Anti-mouse Ig-fluorescein $F(ab')_2$ fragment, Boehringer Mannheim) in RPMI medium containing 0.1% sodium azide and 10% fetal calf serum. Stained cells were supplemented with 2 μg per ml of propidium iodide (Sigma) in PBS and subsequently analyzed on a Becton Dickinson FACScan. The percentage of viable $CD14^+$ monocytes was determined by exclusion of propidium iodide. Granulocyte viability was determined after treatment with A23187 for 10 min in the presence or absence of inhibitors in PBS (0.9 mM Ca^{2+}). Forward and right angle scatter profiles were used for granulocyte gating. The colorimetric WST-1 cytotoxicity assay (Boehringer Mannheim) was performed in U937 cells according to the manufacturer's instructions.

2.8. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Human peripheral blood mononuclear cells (1×10^6 per ml) were stimulated in cell culture medium with 100 ng/ml lipopolysaccharide in the presence or absence of

inhibitors (final concentration 10 μg/ml) for 24 h. Total RNA was extracted with Trisolv (Biotecx) from 1×10^7 cells. For semi-quantitative estimation of cytokine mRNA content, serial 10-fold dilutions of total RNA (Lagoo-Deenadayalan et al., 1993) were reverse transcribed with Oligo-dT_{15} primer (Boehringer Mannheim) for β-actin and with the downstream primer for interleukin-1β (DM156, Perkin Elmer), using the Geneamp-rTth Reverse Transcriptase RNA-PCR-Kit (Perkin Elmer). Subsequent amplification of cDNA by PCR was performed with the interleukin-1β upstream primer (DM155, Perkin Elmer) and β-actin primer pair (Stratagene), according to the manufacturer's instructions (40 × cycles). Amplification products were analyzed by ethidium bromide gel electrophoresis and quantified by video-based densitometry (EASY plus Rev. 3.08 C, Herolab).

2.9. In vivo testing of antiphlogistic effects

All animal experiments were performed according to national and international guidelines. Rats were housed in propylene cages with special animal feed and water ad libitum and maintained on a 12-h light/12-h dark cycle in a temperature-controlled room (21–25°C, 55–70% air humidity). Animals were supplied by a professional breeder and distributor (Charles River Wiga). Adjuvant arthritis was induced in male Lewis rats (200–230 g), according to the method of Newbould (1963), by a single intradermal injection of 100 μl complete Freund's adjuvant containing 0.1 mg inactivated *Mycobacterium tuberculosis* (Difco Laboratories) in 100 μl incomplete Freund's adjuvant (Sigma) into the plantar surface of the right hindpaw between the 2nd and 3rd toes at day 1. Development of inflammation in the left paws not injected with complete Freund's adjuvant as a measure of the secondary immunologic lesion was quantified every second or third day, starting at day 10, by hydroplethysmometry (device of TSE, Bad Homburg, Germany). Control measurements were taken at day 1. To evaluate the immunologic inflammatory reactions within the treatment period, the differences between the volumes measured at day 10 and the following days were calculated. From day 10 up to day 22, i.e., on a therapeutic dosing regimen, animals were treated with inhibitors of secretory phospholipase A_2 or cytosolic phospholipase A_2 or control substances. Drugs were applied by the intraperitoneal or oral route, as indicated in Section 3. One vehicle-treated arthritic group served as treatment control (vehicle control), and another group of healthy animals receiving neither complete Freund's adjuvant injection nor treatment served as control for arthritis induction (naive control). Each group (vehicle control, naive control, treatment groups) consisted of 6 animals.

2.10. Statistical evaluations

Data are expressed as means ± S.E.M. for animals or means ± S.D. for in vitro studies as indicated in the text and legends to figures. Significances were analyzed either by Student's *t*-test for mean differences, assuming unequal variances, or by analysis of variance (ANOVA, two factors with replication).

3. Results

3.1. Inhibitors of cytosolic but not secretory phospholipase A_2 block A23187-induced surface expression of Mac-1 on human peripheral blood granulocytes

Activation of phospholipase A_2 activity followed by the release of lysophospholipids and arachidonic acid and the production of eicosanoids and platelet activating factor can be elicited by treatment of granulocytes with diverse agents including phorbol ester, *N*-formyl-methionyl-leucyl-phenylalanine and Ca^{2+} ionophore A23187. Since phospholipase A_2 activity is reported to be associated with neutrophil degranulation and attachment (O'Flaherty et al., 1983; Tonnensen et al., 1989), we tested the effect of isoform-specific phospholipase A_2 inhibitors on the mobilization of the integrin Mac-1 (CD11b/CD18) from intracellular stores in human peripheral blood granulocytes. In our study, we used different classes of chemical structures, which proved to be inhibitors of either cytosolic phospholipase A_2 or secretory phospholipase A_2, in the corresponding in vitro enzyme assay (Fig. 1) (Tibes et al., 1995). Evaluation of compounds was performed with human recombinant secretory phospholipase A_2 and purified cytosolic phospholipase A_2 from the U937 cell line. The inhibitors of cytosolic phospholipase A_2, 1,1,1-trifluoroheneicosa-6,9,12,15-tetraen-2-one (BM 16.2353, IC_{50} = 0.3 μM) (Street et al., 1993), 1,1,1-trifluoro-nonadeca-10,13,16-trien-2-one (BM 16.2354, IC_{50} = 0.9 μM) and 1,1,1-trifluoro-nonadeca-10,13-dien-2-one (BM 16.2374, IC_{50} = 0.2 μM), represent trifluormethylketone analogues of arachidonic acid C20:4-$COCF_3$, γ-linolenic acid C18:3-$COCF_3$ and linoleic acid C18:2-$COCF_3$. These compounds did not affect secretory phospholipase A_2 activity. The coumarin derivatives 6,7-dihydroxy-4-(4-methoxy-piperidin-1-ylmethyl)-chromen-2-one hydrochloride (BM 16.2056, IC_{50} = 4.1 μM) and (6,7-dihydroxy-2-oxo-2*H*-chromen-4-ylmethylsulfanyl)-acetic acid (BM 16.2266, IC_{50} = 3.5 μM) and the stilbazol derivative 2-[4-(2-pyridin-2-yl-vinyl)-benzylsulfanyl]-benzoic acid sodium salt (BM 16.2287, IC_{50} = 13.5 μM) inhibited secretory phospholipase A_2 without having any effect in the cytosolic phospholipase A_2 assay. NSAIDs (non-steroidal antiinflammatory drugs) including acetylsalicylate and diclofenac, both inhibitors of cyclooxygenases, and tenidap, a dual 5′-lipoxygenase and cyclooxygenase inhibitor, as well as dexamethasone exhibited no inhibitory effect on secretory or cytosolic phospholipase A_2 activity. Flow cytometric analysis of A23187-challenged human peripheral blood granulocytes (Fig. 2) revealed that cytosolic

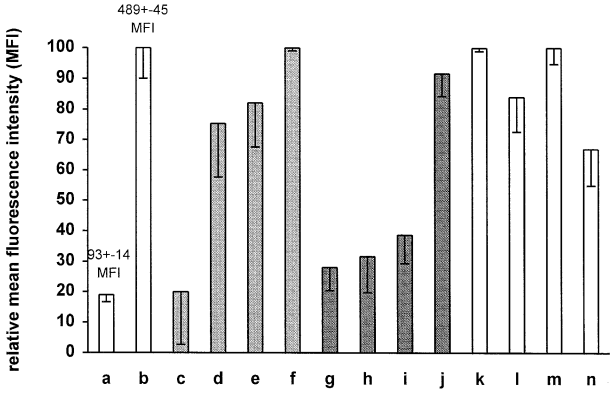

Fig. 2. Inhibition of A23187-induced expression of Mac-1 (CD11b/CD18) on human peripheral blood granulocytes by cytosolic phospholipase A_2 inhibitors. Granulocytes (2×10^6/ml) were preincubated for 15 min with compounds at the concentrations indicated and stimulated by 10 nM A23187 for 10 min. Cells were stained with anti-Mac-1 (CD11b/CD18) for flow cytometry analysis. Values of mean fluorescence intensity (MFI), i.e., Mac-1^+ cells in the granulocyte gate, ranged from 93 ± 14 (a) for unstimulated control granulocytes to 489 ± 45 (b) (was set 100% relative MFI) for ionophore-challenged granulocytes. Values were calculated as mean percent inhibition $\pm$ S.D. compared to stimulated control ($n=3$ different donors). Inhibitors of cytosolic phospholipase A_2 (10, 3, 1, 0.1 μg/ml): (c–f) BM 16.2354, (g–j) BM 16.2374. Inhibitors of secretory phospholipase A_2 (10 μg/ml): (k) BM 16.2266, (l) BM 16.2287. NSAIDs (10 μg/ml): (m) diclofenac, (n) tenidap.

phospholipase A_2 inhibitors of the trifluormethylketone species concentration dependently reduced the surface expression of Mac-1 molecules, with IC_{50} values of 18.9 μM for BM 16.2354 and 2.44 μM for BM 16.2374. Mean fluorescence intensity, corresponding to the number of surface-bound Mac-1, was reduced by 60–100% at an inhibitor concentration of 10 μg/ml compared to that of A23187-stimulated cells. In contrast, neither secretory phospholipase A_2 inhibitors nor the NSAIDs diclofenac and tenidap revealed any inhibitory effect on Mac-1 expression. Drug or vehicle ($\leq 0.1\%$ DMSO) alone had no effect on Mac-1 expression. In order to exclude a generalized suppressive effect of cytosolic phospholipase A_2 inhibitors on surface molecule expression, we analyzed the expression of the granulocyte-specific differentiation marker CDw65 (fucoganglioside receptor) (Macher et al., 1988). In contrast to the marked up-regulation of Mac-1 by Ca^{2+} ionophore treatment, CDw65 is constitutively expressed and neither stimulated by A23187 nor suppressed by cytosolic phospholipase A_2 inhibitors (data not shown). To rule out masking of the antigen by the compounds, we stimulated granulocytes with A23187 in the absence of inhibitors for 10 min. Thereafter cells were washed and inhibitors were added 10 min before antibody staining. None of the substances had a marked inhibitory effect on Mac-1 surface detection (data not shown). To further exclude cytotoxic effects of the compounds, granulocyte viability was determined by flow cytometry, using propidium iodide exclusion. Granulocytes were incubated in the presence of 10 μg/ml of inhibitors for 15 min and stimulated with A23187 for 10 min and subsequently analyzed by flow cytometry. The compound BM 16.2353, the standard cytosolic phospholipase A_2 inhibitor described by Street et al. (1993), was cytotoxic and was not used in further studies. However, none of the other compounds affected the viability of granulocytes (data not shown). These results indicate that these cytosolic phospholipase A_2 inhibitors do not exert their suppressive effect on Mac-1 expression by interfering with the epitope binding site of the antibody or by having unspecific cytotoxic effects, but rather interrupt the intracellular signalling cascade, preventing exocytosis of Mac-1 molecules.

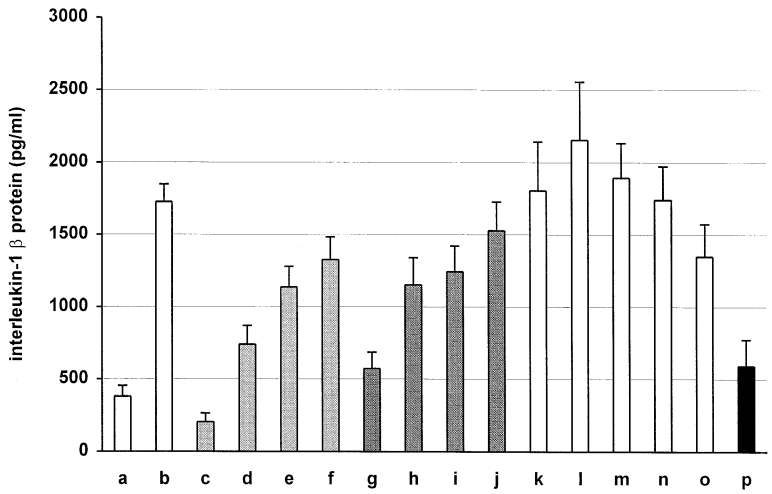

Fig. 3. Suppression of lipopolysaccharide-induced interleukin-1β release from human peripheral blood mononuclear cells by cytosolic phospholipase A_2 inhibitors. Mononuclear cells (10^6/ml) were incubated in the absence (a) and presence of 100 ng/ml lipopolysaccharide (b) for 24 h with compounds at the final concentrations indicated. Inhibitors of cytosolic phospholipase A_2 (10, 3, 1, 0.1 μg/ml): (c–f) BM 16.2354, (g–j) BM 16.2374. Inhibitors of secretory phospholipase A_2 (10 μg/ml): (k) BM 16.2056, (l) BM 16.2266. NSAIDs (10 μg/ml): (m) acetylsalicylate, (n) tenidap, (o) diclofenac. Steroid (10 μg/ml): (p) dexamethasone. Interleukin-1β was quantified in the supernatants by ELISA. Values are expressed as means $\pm$ S.E.M. ($n\geq6$ different donors). Significance was analyzed by *t*-test: (c),(d),(g) = $P<0.001$; (e),(h) = $P<0.01$.

3.2. Inhibitors of cytosolic but not secretory phospholipase A_2 suppress lipopolysaccharide-stimulated release of interleukin-1β from human peripheral blood mononuclear cells

Stimulation of human peripheral blood monocytes with lipopolysaccharide induces the expression and activity of phospholipase A_2 enzymes, followed by the release of arachidonic acid and the production of eicosanoids and platelet activating factor, which themselves are potent inducers of the synthesis of proinflammatory cytokines. To decide which isoform is involved in this signalling pathway, we tested isoform-specific phospholipase A_2 inhibitors on lipopolysaccharide-induced synthesis of interleukin-1β, tumor necrosis factor-α and interleukin-6 in peripheral blood mononuclear cells from healthy donors. Donors were anonymous, randomly selected, and confirmed not to be receiving medical treatment and did not have any allergic or chronic inflammatory diseases. Average cytokine levels in supernatants of lipopolysaccharide-challenged (100 ng/ml for 24 h) human blood mononuclear cells were 1.5 ± 0.8 ng/ml for interleukin-1β, 15.7 ± 9.6 ng/ml for interleukin-6 and 0.5 ± 0.6 ng/ml for tumor necrosis factor-α ($n = 20$ different donors). High interindividual variability of cytokine production was recorded. We distinguished between low and high responders to lipopolysaccharide in whom interleukin-1β levels reached 0.8 ± 0.3 ng/ml and 2.1 ± 0.5 ng/ml, respectively. Despite careful donor selection, some individuals already displayed high interleukin-1β levels in the absence of lipopolysaccharide stimulation and were termed 'prestimulated' donors. Their basal interleukin-1β levels were 0.9 ± 0.4 ng/ml compared to 6 ± 14 pg/ml in naive donors. We found that the cytosolic phospholipase A_2 inhibitors of the trifluormethylketone species concentration dependently (Fig. 3) reduced protein levels of interleukin-1β in the supernatants of lipopolysaccharide-stimulated human blood mononuclear cells after an incubation of 24 h. IC_{50} values were 14.8 μM for BM 16.2353, 11.8 μM for BM 16.2354 and 19.0 μM for BM 16.2374 ($n = 8$). The latter two trifluormethylketones at a concentration of 10 μg/ml differentially down-regulated interleukin-1β release to $91 \pm 12\%$ and $66 \pm 19\%$ ($n = 8$), respectively, but did not affect levels of interleukin-6 and tumor necrosis factor-α. This finding indicated that these two trifluormethylketones act selectively on interleukin-1β synthesis. In contrast, the standard compound BM 16.2353, at a concentration of 10 μg/ml, abolished the production of all three cytokines by 80–100%, pointing at a cytotoxic potential (see later in this section). Dexamethasone markedly reduced interleukin-1β release by 60%. In contrast, the secretory phospholipase A_2 inhibitors BM 16.2056 and BM 16.2266 as well as the NSAIDs acetylsalicylate, tenidap and diclofenac did not suppress interleukin-1β release. The solvent ($\leq 0.1\%$ DMSO) alone exerted no effect on lipopolysaccharide-induced cytokine production. We found no difference between the inhibition values for interleukin-1β production in the above-mentioned subclasses of donors, i.e., prestimulated subjects as well as high and low responders to lipopolysaccharide were equally affected by the compounds. We further investigated the effect of these compounds on the viability of peripheral blood mononuclear cell subpopulations. Previous studies revealed that none of the substances used in this study exerted a cytotoxic effect on mitogen-activated T- and B-lymphocytes, as shown by flow cytometry-based cell cycle analysis (Rodewald, 1995). To determine monocyte viability, human blood mononuclear cells were stimulated with lipopolysaccharide in the presence of increasing concentrations of inhibitors for 24 h. Subsequently, cells were stained with anti-CD14 and analyzed by flow cytometry, using propidium iodide exclusion (data not shown). The secretory phospholipase A_2 inhibitors and the NSAIDs acetylsalicylate, diclofenac and tenidap as well as dexamethasone had no cytotoxic effect on monocytes. The trifluormethylketones BM 16.2354 and BM 16.2374 exhibited no or only weak cytotoxicity against $CD14^+$ monocytes. The proportion of dead or degraded monocytes after 24-h incubation with 10 μg/ml inhibitor compared to that of lipopolysaccharide-stimulated control cells was 20% for BM 16.2354 and 0% for BM 16.2374. At concentrations of 1 μg/ml, no toxic effects on monocytes were observed. In contrast, the arachidonoyl derivative BM 16.2353 reduced the number of monocytes to 40% of control. Consequently, we did not use this compound in further investigations.

3.3. Inhibitors of cytosolic phospholipase A_2 reduce synthesis of interleukin-1β protein in human peripheral blood mononuclear cells and PMA-differentiated monocytic U937 cells

In the following experiments, intracellular cytokine levels were determined by flow cytometry. Human peripheral blood mononuclear cells were stimulated with lipopolysaccharide in the presence of 10 μg/ml inhibitor for 2–24 h. Subsequently, monocytes were double-stained with anti-interleukin-1β and anti-CD14. Expression of intracellular interleukin-1β was detectable 2 h after lipopolysaccharide stimulation and markedly increased within 4 h. Furthermore, the monocyte population was still dense after 4 h of lipopolysaccharide stimulation, resulting in precise gating during flow cytometric analysis. Therefore, analysis of the effect of compounds on interleukin-1β expression in monocytes was performed after a 4-h incubation. We found that in human $CD14^+$ peripheral blood monocytes the cytosolic phospholipase A_2 inhibitors BM 16.2354, BM 16.2374 and dexamethasone suppressed the expression of interleukin-1β protein at the single cell level, whereas acetylsalicylate and the secretory phospholipase A_2 inhibitors were ineffective. The proportion of double-positive cells declined by $46 \pm 14\%$ for BM 16.2354 and $53 \pm 21\%$ for BM 16.2374 compared to lipopolysaccha-

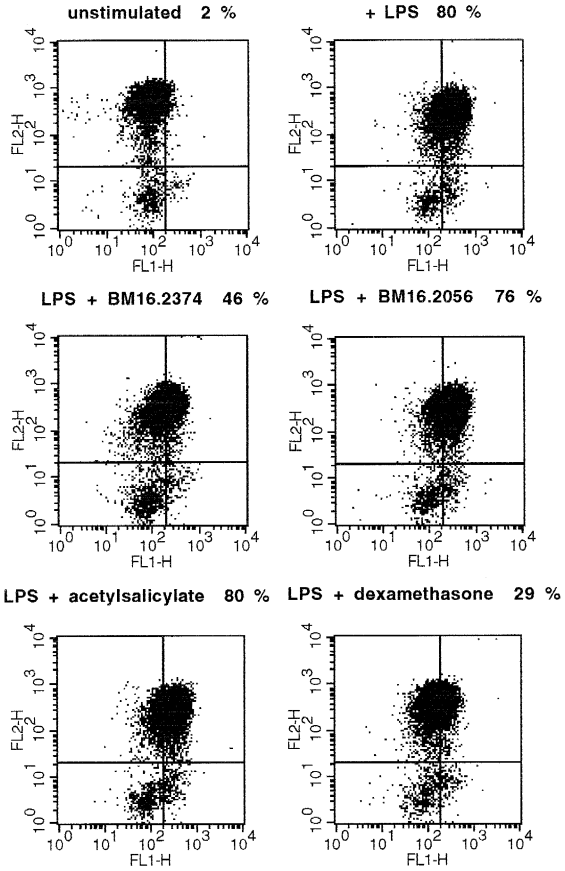

Fig. 4. Suppression of intracellular interleukin-1β synthesis by an inhibitor of cytosolic phospholipase A_2 (BM 16.2374) but not by an inhibitor of secretory phospholipase A_2 (BM 16.2056) in lipopolysaccharide-stimulated human peripheral blood $CD14^+$ monocytes. Peripheral blood mononuclear cells (10^6/ml) were stimulated with 100 ng/ml lipopolysaccharide (LPS) with the compounds at a final concentration of 10 μg/ml for 4 h. Cells were fixed and stained with anti-interleukin-1β, followed by FITC-labeled detection antibody and PE-labeled anti-CD14 and analyzed by flow cytometry. Fluorescence 1 (FITC) representing interleukin-1β signal is plotted against fluorescence 2 (PE) representing $CD14^+$ monocytes. The percentage of double-positive cells in the upper right quadrant compared to total $CD14^+$ cells in the upper two quadrants is indicated. One representative experiment is shown ($n = 8$ different donors).

ride-challenged controls after a stimulation period of 4 h ($n = 8$ different donors). Fig. 4 displays a representative flow cytometric dot blot illustrating the about 50% decrease in the proportion of interleukin-1β protein expressing blood monocytes from a healthy donor after treatment with BM 16.2374. In order to circumvent donor variability, we additionally investigated cells from the PMA-differentiated monocytic U937 cell line. Undifferentiated promonocytic U937 cells did not express cytokines upon lipopolysaccharide stimulation. However, after treatment with PMA for 3 days U937 cells displayed a macrophage phenotype (Wiederhold et al., 1988), which was identified by marked up-regulation of the macrophage-specific surface marker Mac-1 (Grattage et al., 1992). These cells efficiently responded to lipopolysaccharide stimulation with the release of interleukin-1β (0.3 ± 0.1 ng/ml), as evaluated by enzyme-linked immunosorbent assay (ELISA) ($n = 6$). The ability of the U937 cell line to metabolize arachidonic acid and produce leukotrienes and prostaglandins after stimulation by lipopolysaccharide, PMA and ionophore increases during differentiation into macrophage-like cells (Wiederhold et al., 1988). We therefore chose this cell line to

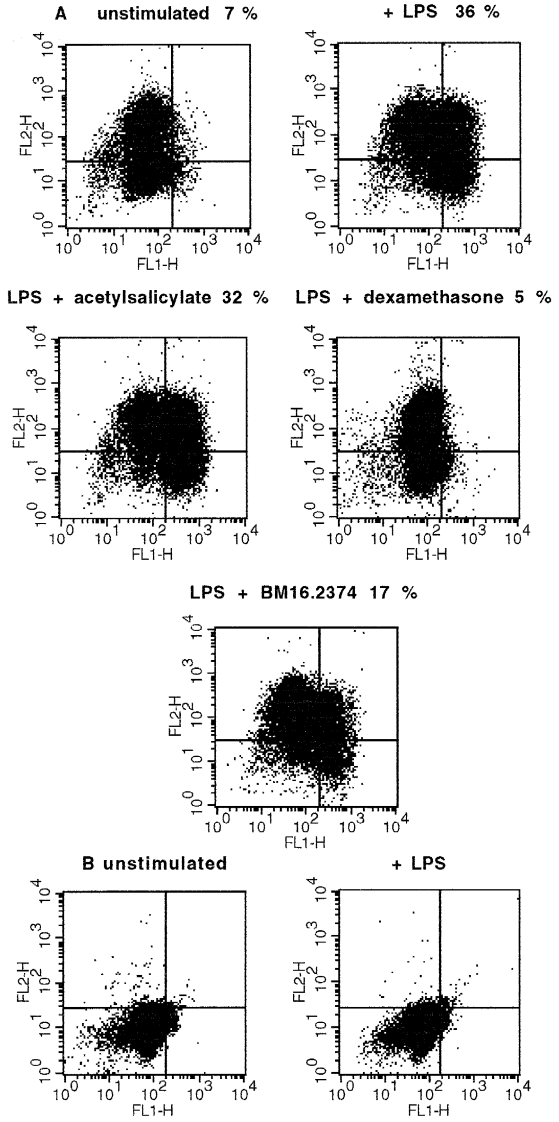

Fig. 5. Suppression of intracellular interleukin-1β synthesis in PMA-differentiated U937 cells by an inhibitor of cytosolic phospholipase A_2 (BM 16.2374). Panel A: PMA-differentiated U937 cells (10^6/ml) were stimulated with 100 ng/ml lipopolysaccharide (LPS) in the presence of compounds at a final concentration of 10 μg/ml for 24 h. Panel B: Undifferentiated cells (10^6/ml) were treated with lipopolysaccharide. Thereafter cells were fixed and stained with anti-interleukin-1β, followed by FITC-labeled detection antibody and PE-labeled anti-Mac-1 as a marker of differentiation and analyzed by flow cytometry. Fluorescence 1 (FITC) representing intracellular interleukin-1β is plotted against fluorescence 2 (PE) representing $Mac\text{-}1^+$ macrophage-like cells. Panel A: The percentage of double-positive cells in the upper right quadrant compared to total is indicated. One representative experiment is shown ($n = 3$).

further investigate the molecular effect of cytosolic phospholipase A_2 inhibitors. Intracellular expression of interleukin-1β was effectively suppressed in differentiated cells by dexamethasone and the trifluormethylketones BM 16.2354 ($51 \pm 14\%$) and BM 16.2374 ($44 \pm 15\%$) (Fig. 5A) at a concentration of 10 μg/ml compared to lipopolysaccharide-stimulated controls ($n = 3$). The secretory phospholipase A_2 inhibitors and acetylsalicylate were ineffective. Undifferentiated U937 cells were not able to express interleukin-1β protein upon lipopolysaccharide stimulation (Fig. 5B). Levels of released interleukin-1β protein in supernatants from lipopolysaccharide-stimulated, differentiated U937 cells were reduced after treatment with the cytosolic phospholipase A_2 inhibitors BM 16.2354 ($70 \pm 1\%$) and BM 16.2374 ($57 \pm 13\%$), as determined by ELISA ($n = 4$) (Fig. 6). No cytotoxic effects of these compounds were recorded over 24 h at an inhibitor concentration of 10 μg/ml (data not shown). These results indicate that biosynthesis of interleukin-1β but not secretion of the cytokine is affected by cytosolic phospholipase A_2 inhibitors.

3.4. Inhibitors of cytosolic phospholipase A_2 reduce interleukin-1β mRNA steady-state level in human peripheral blood mononuclear cells

To further elucidate the molecular mechanism of cytokine suppression, we tested the effect of cytosolic phospholipase A_2 inhibitors on the interleukin-1β mRNA steady-state level in human peripheral blood mononuclear cells. At a concentration of 10 μg/ml, the trifluormethylketones BM 16.2374 (Fig. 7) and BM 16.2354 (not shown) reduced the steady-state level of mRNA for interleukin-1β, determined 24 h after lipopolysaccharide challenge. In contrast, the NSAID acetylsalicylate had no effect on interleukin-1β mRNA levels. Video-based densitometry of the PCR products in gels (see far right lanes of gel in Fig. 7) revealed a reduction in the amount of interleukin-1β mRNA by 60% compared to that of lipopolysaccharide-stimulated control cells, whereas the level of mRNA for β-actin remained unaffected by the inhibitor.

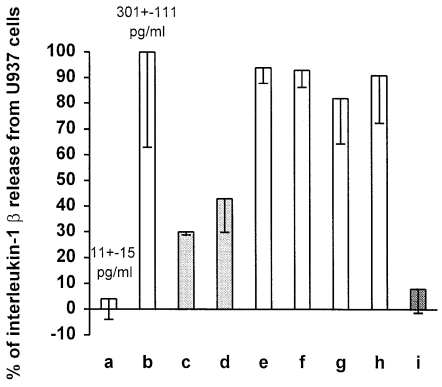

Fig. 6. Reduction of lipopolysaccharide-induced interleukin-1β release from PMA-differentiated U937 cells by cytosolic phospholipase A_2 inhibitors. Cells were cultured with PMA for 3 days, reseeded (10^6/ml) in the absence (a) and presence of 100 ng/ml lipopolysaccharide (b) and compounds at a final concentration of 10 μg/ml for 24 h. Inhibitors of cytosolic phospholipase A_2: (c) BM 16.2354, (d) BM 16.2374. Inhibitors of secretory phospholipase A_2: (e) BM 16.2056, (f) BM 16.2266, (g) BM 16.2287. NSAID: (h) acetylsalicylate. Steroid: (i) dexamethasone. Interleukin-1β was quantified in the supernatants by ELISA. Data are expressed as mean percent inhibition ± S.D. compared to lipopolysaccharide-stimulated control ($n = 4$).

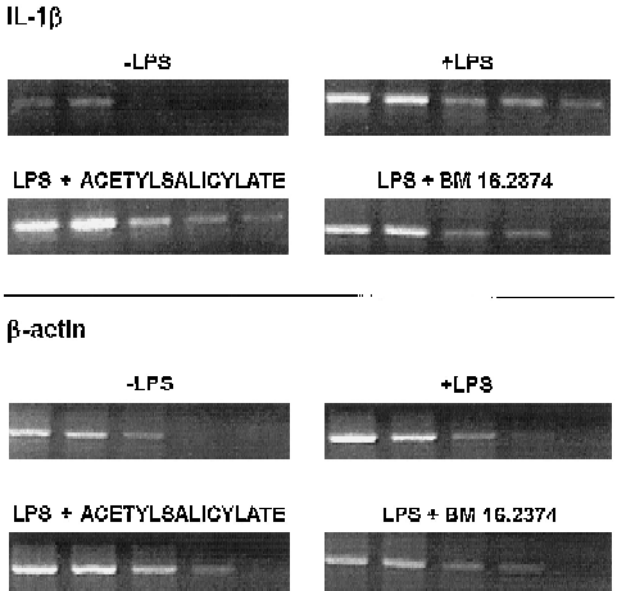

Fig. 7. Effect of an inhibitor of cytosolic phospholipase A_2 (BM 16.2374) on lipopolysaccharide-induced steady-state level of interleukin-1β mRNA in human peripheral blood mononuclear cells. Cells (10^6/ml) were stimulated with 100 ng/ml lipopolysaccharide (LPS) in the presence or absence of inhibitors at a final concentration of 10 μg/ml for 24 h. Total RNA was extracted from 10^7 cells. For semi-quantitative estimation of mRNA levels 10-fold serial dilutions of total RNA (lanes 1–5 represent dilutions 10^0 to 10^{-4}) were used for reverse transcription followed by PCR, yielding amplification fragments for interleukin-1β (391 bp) and β-actin (661 bp). One experiment out of three similar experiments is shown.

3.5. Inhibitors of cytosolic but not secretory phospholipase A_2 ameliorate chronic inflammation in vivo

Substances which were identified as cytosolic phospholipase A_2 or secretory phospholipase A_2 inhibitors were subsequently tested for their in vivo efficacy against inflammation. To evaluate the antiphlogistic effect of compounds on T-cell-mediated chronic inflammation, the rat model of adjuvant arthritis was used. All tested cytosolic phospholipase A_2 inhibitors ameliorated the progression of immunologic inflammation, with inhibition values ranging between 60% and 100%, resembling the effects of orally administered dexamethasone (Fig. 8). No significant differences between cytosolic phospholipase A_2 inhibitors and dexamethasone were recorded. In contrast to cytosolic phospholipase A_2 inhibitors, the tested secretory phospholipase A_2 inhibitors were completely ineffective in this

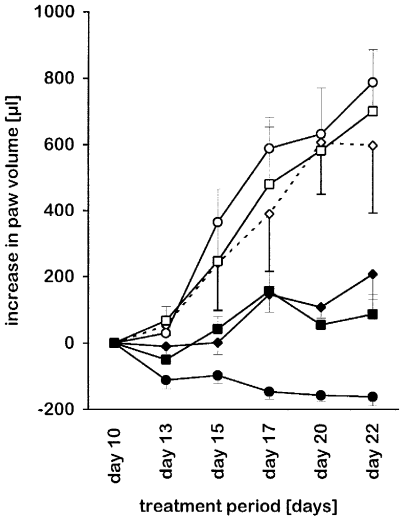

Fig. 8. Inhibition of the T-cell-mediated component of adjuvant arthritis in Lewis rats by inhibitors of cytosolic phospholipase A_2 but not secretory phospholipase A_2. Injection of CFA was on day 1. Compounds were given between day 10 and 22 after complete Freund's adjuvant injection: (◇) vehicle; (●) dexamethasone, 1 mg/kg p.o.; inhibitors of secretory phospholipase A_2: (□) BM 16.2056, 20 mg/kg i.p.; (○) BM 16.2266, 20 mg/kg i.p.; inhibitors of cytosolic phospholipase A_2: (◆) BM 16.2354, 20 mg/kg i.p.; (■) BM 16.2374, 20 mg/kg i.p. All phospholipase A_2 inhibitors were tolerated by the animals without any side effect. Paw volumes of secondary lesions were measured on the days indicated. Values represent means ± S.E.M. ($n = 6$ per group). Significance ($P < 0.001$ for all compounds) was tested with ANOVA.

model of T-cell-mediated inflammation. This finding, together with those of the in vitro studies, indicates an essential function of cytosolic phospholipase A_2 in the development of chronic inflammation.

4. Discussion

The data presented in this study demonstrate that phospholipase A_2 plays a prominent role in inflammation. Based on in vitro and in vivo experiments, we provide evidence that cytosolic phospholipase A_2 but not secretory phospholipase A_2 triggers and amplificates the inflammatory cascade.

The trifluormethylketone analogue of arachidonic acid C20:4-$COCF_3$ (analogue of AA-$COCF_3$) is a potent, slow binding inhibitor of cytosolic phospholipase A_2 and has a 500-fold greater potency against cytosolic than against secretory phospholipase A_2 (Street et al., 1993), reversibly binding to the active site of the enzyme (Trimble et al., 1993). This inhibitor prevents arachidonic acid release in human platelets stimulated with Ca^{2+} ionophore (Riendeau et al., 1994) or thrombin (Bartoli et al., 1994), whereas secretory phospholipase A_2 inhibitors are ineffective in this context. In platelets cytosolic phospholipase A_2 is 100-fold more abundant than secretory phospholipase A_2 and is phosphorylated within minutes after stimulation with thrombin (Kramer et al., 1993). Together, these studies argue that AA-$COCF_3$ is acting as a specific inhibitor of cytosolic phospholipase A_2, thereby supporting a prominent role of this enzyme in arachidonic acid release. We have synthesized AA-$COCF_3$ (BM 16.2353) as a reference compound and its two analogues γ-linolenoyl trifluormethylketone 18:3-$COCF_3$ (BM 16.2354) and linoloyl trifluormethylketone 18:2-$COCF_3$ (BM 16.2374) (for structures and full chemical names see Fig. 1). All three trifluormethylketones inhibited cytosolic phospholipase A_2 activity with no measurable effect on secretory phospholipase A_2. To elucidate the role of the two phospholipase A_2 isoforms we tested these compounds in cellular assays reflecting different temporal stages of the inflammatory cascade. Although a general consensus exists regarding the importance of cytosolic phospholipase A_2 in arachidonic acid release, a possible contribution of secretory phospholipase A_2 cannot be ruled out (Barbour and Dennis, 1993). We found that none of the secretory phospholipase A_2 inhibitors were able to prevent the release of arachidonic acid from collagen-challenged human platelets. However, at a concentration of 10 μg/ml the three trifluormethylketones BM 16.2353 (70%), BM 16.2354 (50%) and BM 16.2374 (38%) diminished arachidonic acid release (unpublished observation). We have shown previously that the trifluormethylketones at a concentration of 10 μg/ml are efficient in blocking leukotriene B_4 and prostaglandin E_2 synthesis by more than 80% in Ca^{2+} ionophore-stimulated human peripheral blood mononuclear cells (Tibes et al., 1995). The trifluormethylketones did not affect 5′-lipoxygenase or cyclooxygenase activity, as assayed by arachidonic acid-induced leukotriene C_4 and prostaglandin E_2 synthesis in human leukocytes and in crude enzyme preparations from human leukocytes (Tibes et al., 1997). These results indicated that these cytosolic phospholipase A_2 inhibitors act specifically to prevent the initiation of the arachidonic acid signalling cascade. However, unspecificity cannot be ruled out unless other phospholipid metabolizing enzymes, e.g., phospholipase C and D, are taken into consideration (work in progress). Besides the primary effects of cytosolic phospholipase A_2 inhibitors, we were interested in additional antiinflammatory modes of action of these compounds. First, we evaluated whether a correlation exists between phospholipase A_2 inhibition and surface expression of the β_2-integrin Mac-1. The initial phase of cellular infiltration involves leukocytes rolling along the endothelium, mediated by members of the selectin family. However, this event does not lead to firm attachment and diapedesis unless the β_2-integrins are engaged. In contrast to LFA-1 (leukocyte function associated antigen-1, CD11a/CD18) and p150,95 (CD11c/CD18), Mac-1 (CD11b/CD18) seems to play a major role because it is released within minutes upon stimulation by leukotriene B_4 and platelet activating factor (Miller et al., 1987; Horie and Kita, 1994). Since human neutrophils express both phospholipase A_2 isoforms (Marshall and Roshak, 1993), modulation of Mac-1 surface expression by

phospholipase A_2 inhibitors should further clarify the role of secretory phospholipase A_2 versus cytosolic phospholipase A_2 in this early inflammatory event. We found that neither the secretory phospholipase A_2 inhibitors nor the NSAIDs diclofenac, a cyclooxygenase inhibitor, and tenidap, a dual blocking agent of cyclooxygenase and 5′-lipoxygenase, exerted a suppressive effect on Ca^{2+} ionophore-induced Mac-1 surface expression in human peripheral blood granulocytes. However, the cytosolic phospholipase A_2 inhibitors of the trifluormethylketone species in μM concentrations reduced the expression of this integrin by 60–100%. Recent studies revealed that unselective phospholipase A_2 inhibitors weaken the adherence and spreading of macrophages as well as diapedesis and degranulation of neutrophils (Perretti and Flower, 1993). Jacobson and Schrier (1993) confirmed that phospholipase A_2 regulates Mac-1 expression in human neutrophils. Inhibitors of cyclooxygenases, 5′-lipoxygenases or protein kinases did not modulate Mac-1 expression, whereas manoalide, scalaradial and *para*-bromphenylacyl bromide suppressed it. However, it is difficult to attribute the effect of these 'classical' phospholipase A_2 inhibitors to specific inhibition of phospholipase A_2 activity alone. The unspecificity of manoalide has been documented because the compound suppresses phospholipase C, ornithine decarboxylase and 5′-lipoxygenase (Glaser, 1995). Scalaradial interferes with calcium mobilization and inositol formation, indicating that it exerts additional pharmacological actions (Barnette et al., 1994). *Para*-bromphenylacyl bromide nonspecifically inhibits various enzymes (phospholipase A_2 and C) by covalent binding to histidine residues (Kyger and Franson, 1984). None of the substances exerts a prominent inhibitory effect on cytosolic phospholipase A_2 (Winkler et al., 1994). In contrast, the compounds applied in this study prevented Mac-1 expression by blocking the enzymatic activity of cytosolic phospholipase A_2. Since Mac-1 is stored in specific granules, cytosolic phospholipase A_2 inhibitors may prevent surface expression by blocking exocytosis. *Cis*-unsaturated fatty acids support membrane fusion by activation of fusion proteins like synexins (Meers et al., 1988), and lysophospholipids are known to facilitate degranulation of histamine from triggered mast cells (Murakami et al., 1992). We therefore propose that cytosolic phospholipase A_2 inhibitors interfere with signal transduction leading to triggered exocytosis of membrane vesicles by cutting off the supply of fusogenic lipid mediators. Consequently, by down-regulating adhesion molecule expression on granulocytes, namely neutrophils, cytosolic phospholipase A_2 inhibitors may be beneficial in inflammation by reducing cellular infiltration and necrotic damage in the affected tissue.

In human leukocytes lipopolysaccharide induces the activity of both secretory phospholipase A_2 and cytosolic phospholipase A_2, with subsequent production of eicosanoids and platelet activating factor (Mohri et al., 1990; Vadas et al., 1993; Doerfler et al., 1994). Arachidonic acid (Baldie et al., 1993), leukotrienes B_4 and D_4 (Rola-Pleszczynski and Lemaire, 1985; Porreca et al., 1995) and platelet activating factor (Barthelson and Valone, 1991; Poubelle et al., 1991) are reported to enhance interleukin-1β synthesis, whereas contradictory data exist about modulation of interleukin-1β expression by prostaglandin E_2 (Zhong et al., 1995). After cytokine synthesis further activation and synthesis of phospholipase A_2 occurs, establishing superinduction of the inflammatory cascade. In this cascade, interleukin-1β itself is a potent inducer of the expression and activity of phospholipase A_2 enzymes in an autocrine and paracrine fashion (Vadas et al., 1993). Moreover, leukotrienes B_4 and D_4 and platelet activating factor positively influence the activity of phospholipase A_2 (Clark et al., 1986; Nigam et al., 1995; Reddy et al., 1995; Wijkander et al., 1995). This sequence of events indicates that there is a self-perpetuating positive feedback loop (Tibes et al., 1993; Wijkander et al., 1995), which may convert an acute inflammatory attack into a chronic stage. Accordingly, it seemed reasonable that phospholipase A_2 inhibitors are potent in short-cutting this autoregulatory loop. In order to define the relevant phospholipase A_2 isoform in this process, we evaluated whether secretory or cytosolic phospholipase A_2 inhibitors modulate cytokine expression. Indeed, our data show that the cytosolic phospholipase A_2 inhibitors of the trifluormethylketone species prevented lipopolysaccharide-induced synthesis of interleukin-1β in human peripheral blood mononuclear cells by 60–90%, whereas secretory phospholipase A_2 inhibitors and NSAIDs were ineffective in this context. This finding is consistent with our previous studies, which revealed that in human blood mononuclear cells cytosolic phospholipase A_2 is expressed much more abundantly than secretory phospholipase A_2. Accordingly, we detected only marginal levels of secretory phospholipase A_2 in the promonocytic cell line U937 in contrast to marked expression of cytosolic phospholipase A_2 (Rodewald et al., 1994). We therefore expected a dominant role of cytosolic phospholipase A_2 over secretory phospholipase A_2 in these cells. The reference compound arachidonoyl trifluormethylketone AA-$COCF_3$ (BM 16.2353) (Street et al., 1993) was not used in our cellular assays due to its marked cytotoxicity. In contrast, the two trifluormethylketones of γ-linolenic (BM 16.2354) and linoleic acid (BM 16.2374) selectively suppressed interleukin-1β synthesis but left interleukin-6 and tumor necrosis factor-α levels as well as monocyte viability unaffected. Inhibition values for lipopolysaccharide-induced interleukin-1β synthesis were comparable in human blood mononuclear cells of both naive donors and 'prestimulated' donors, who already displayed elevated interleukin-1β levels in the absence of lipopolysaccharide stimulation. This points to the therapeutic as well as prophylactic efficacy of these compounds. Intracellular quantification of interleukin-1β in human $CD14^+$ blood monocytes and PMA-differentiated cells of the U937 cell

line by flow cytometry revealed that the trifluormethylketones BM 16.2354, BM 16.2374 and dexamethasone suppressed intracellular biosynthesis of interleukin-1β protein. This indicates that secretion and processing of the cytokine by interleukin-1 converting enzyme remained unaffected. Additional quantification of human 35-kDa pre-interleukin-1β in sonicated cell lysates of lipopolysaccharide-stimulated blood mononuclear cells by ELISA (Cistron Biotechnology) confirmed that levels of pre-interleukin-1β are reduced by these compounds, comparable to their effect on mature 17-kDa interleukin-1β (data not shown). The NSAIDs acetylsalicylate, diclofenac and tenidap did not alter cytokine levels in supernatants of lipopolysaccharide-stimulated blood mononuclear cells. There is now evidence that cyclooxygenase inhibition increases leukotriene B_4 production and conversely lipoxygenase inhibition enhances prostaglandin E_2 output. Accordingly, even blockade of both pathways, e.g., by tenidap, may lead to increased formation of platelet activating factor, lipoxins and hydroxy/peroxy fatty acids, resulting in a metabolic shunting effect. This may explain the failure of NSAIDs to effectively block the inflammatory cascade (Marshall et al., 1991; Wardle et al., 1993; Pettipher and Wimbery, 1994). Further emphasizing a molecular mode for cytokine suppression, we observed that the two trifluormethylketones reduced the steady-state level of mRNA for interleukin-1β in human lipopolysaccharide-stimulated blood mononuclear cells. We therefore propose that cytosolic phospholipase A_2 inhibitors of the trifluormethylketone class interfere with interleukin-1β transcription or mRNA stability. Now ample evidence exists that lipid mediators resulting from phospholipase A_2 activity act on transcription factors. Arachidonic acid induces mRNA for the immediate-early genes egr-1 and c-fos (Danesch et al., 1994). Leukotriene B_4 directly activates preexisting NF-κB and NF-IL6 (Brach et al., 1992), while platelet activating factor stimulates the expression of both c-fos and c-jun, increasing the formation of active AP-1 heterodimers (Squinto et al., 1989). However, the molecular mechanism of action of lipid metabolites and cytosolic phospholipase A_2 inhibitors on gene induction and transcription factor activity remains to be elucidated in further experiments.

In vivo testing revealed that cytosolic phospholipase A_2 plays a prominent role in T cell-mediated chronic inflammation, as reflected by the inhibition of adjuvant arthritis in rat by cytosolic phospholipase A_2 inhibitors, an effect comparable to that elicited by steroid therapy (Croxtall et al., 1995; Hamann et al., 1995). None of the secretory phospholipase A_2 inhibitors ameliorated arthritic inflammation. These data support the hypothesis that elevated secretory phospholipase A_2 activity defines the acute state of an inflammatory response, whereas sustained superinduction of cytosolic phospholipase A_2 constitutes the chronic form of inflammation which may be overlaid by acute attacks additionally involving secretory phospholipase A_2. Referring to our in vitro data, we assume that the beneficial effects of cytosolic phospholipase A_2 inhibitors in chronic inflammation may rely on inhibition of the synthesis of proinflammatory lipid mediators, which is followed by down-regulation of gene expression for adhesion molecules in granulocytes and for cytokines in monocytes and presumably T-lymphocytes because interleukin-1β is a potent modulator of the T-cell response. However, further investigations of the influence of cytosolic phospholipase A_2 inhibitors on leukocyte distribution and migration in arthritic tissue are necessary.

Our studies substantiate the proposal of others (Marshall et al., 1991; Glaser, 1995) that cytosolic phospholipase A_2 might be a novel pharmacological target to be addressed in antiinflammatory therapy. The discrepancy between effective inhibition of secretory phospholipase A_2 and the absence of therapeutic efficacy in a chronic model of inflammation indicate that cytosolic phospholipase A_2 may be the prime enzyme in related diseases. Thus, inhibition of cytosolic phospholipase A_2 holds promise for interfering with the positive feedback loop, thereby reducing cellular infiltration and degradative damage of infected or traumatized tissue. However, despite preclinical data, conclusive results on the dominant role of cytosolic phospholipase A_2 in chronic inflammation can only be provided in clinical studies with appropriate inhibitors.

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