Coupling of boswellic acid-induced Ca²⁺ mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes

[1]Anja Altmann, [1]Daniel Poeckel, [1]Lutz Fischer, [1]Manfred Schubert-Zsilavecz, [1]Dieter Steinhilber & *,[1]Oliver Werz

[1]Institute of Pharmaceutical Chemistry, University of Frankfurt, Marie-Curie Strasse 9, Frankfurt D-60439, Germany

1   We have previously shown that 11-keto boswellic acids (11-keto-BAs), the active principles of *Boswellia serrata* gum resins, activate p38 MAPK and p42/44$^{\text{MAPK}}$ and stimulate Ca²⁺ mobilisation in human polymorphonuclear leucocytes (PMNL).

2   In this study, we attempted to connect the activation of MAPK and mobilisation of Ca²⁺ to functional responses of PMNL, including the formation of reactive oxygen species (ROS), release of arachidonic acid (AA), and leukotriene (LT) biosynthesis.

3   We found that, in PMNL, 11-keto-BAs stimulate the formation of ROS and cause release of AA as well as its transformation to LTs *via* 5-lipoxygenase.

4   Based on inhibitor studies, 11-keto-BA-induced ROS formation is Ca²⁺-dependent and is mediated by NADPH oxidase involving PI 3-K and p42/44$^{\text{MAPK}}$ signalling pathways. Also, the release of AA depends on Ca²⁺ and p42/44$^{\text{MAPK}}$, whereas the pathways stimulating 5-LO are not readily apparent.

5   Pertussis toxin, which inactivates $G_{i/0}$ protein subunits, prevents MAPK activation and Ca²⁺ mobilisation induced by 11-keto-BAs, implying the involvement of a $G_{i/0}$ protein in BA signalling.

6   Expanding studies on differentiated haematopoietic cell lines (HL60, Mono Mac 6, BL41-E-95-A) demonstrate that the ability of BAs to activate MAPK and to mobilise Ca²⁺ may depend on the cell type or the differentiation status.

7   In summary, we conclude that BAs act *via* $G_{i/0}$ protein(s) stimulating signalling pathways that control functional leucocyte responses, in a similar way as chemoattractants, that is, *N*-formyl-methionyl-leucyl-phenylalanine or platelet-activating factor.
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Introduction

Extracts of *Boswellia serrata* gum resins have been traditionally used as folk medicine to cure inflammatory and arthritic diseases (Safayhi & Sailer, 1997). It was found that *B. serrata* extracts suppress the formation of proinflammatory leukotrienes (LTs), and boswellic acids (BAs) were identified as the active principles targeting 5-lipoxygenase (5-LO), the key enzyme in LT biosynthesis (Safayhi *et al.*, 1992; 1995). In addition, human leucocyte elastase was found to be a target for BAs (Safayhi *et al.*, 1997). Aside of these anti-inflammatory

implications, BAs have been reported to influence the growth and differentiation of tumour cells. Thus, *B. serrata* extracts or isolated BAs induce the apoptosis of brain tumour cell lines (Glaser *et al.*, 1999), meningioma cells (Park *et al.*, 2002), rat gliomas (Winking *et al.*, 2000), liver and colon cancer cell lines (Liu *et al.*, 2002a), and also of leukaemic cells (Hoernlein *et al.*, 1999). Caspase-8 (Liu *et al.*, 2002a), topoisomerases (Hoernlein *et al.*, 1999), and the p42/44$^{\text{MAPK}}$ pathway (Park *et al.*, 2002) have been suggested as signalling molecules mediating the apoptotic effects of BAs.

With respect to inhibition of 5-LO, 3-acetyl-11-keto-BA (AKBA) was the most potent BA, whereas BAs lacking an 11-keto-group were weak 5-LO inhibitors (Safayhi *et al.*, 1992).

*Author for correspondence;
E-mail: o.werz@pharmchem.uni-frankfurt.de

The IC_{50} values of AKBA for inhibition of 5-LO differ between different groups and appear to depend also on the cell type. Thus, the IC_{50} values were determined in the range of $1.5\,\mu M$ for 5-LO in rat neutrophils (Safayhi *et al.*, 1992) and $12-15\,\mu M$ in differentiated HL60 and MM6 cells, respectively (Werz *et al.*, 1997). In cell-free systems, the IC_{50} values of AKBA for 5-LO inhibition were around $50\,\mu M$, implying that, in intact cells, cellular components or mechanisms improve the efficacy of AKBA (Werz *et al.*, 1997). Finally, low concentrations of ethanolic extracts of *B. serrata* potentiated 5-LO product formation in PMNL induced by ionophore (Safayhi *et al.*, 2000), and 3-oxo-tirucallic acid, isolated from *B. serrata* extracts, stimulated 5-LO product synthesis in resting and agonist-challenged PMNL (Boden *et al.*, 2001). Therefore, until today, inhibition of 5-LO as the main principle for the anti-inflammatory effects of *B. serrata* extracts, determined in several *in vivo* models (Gupta *et al.*, 1992; Krieglstein *et al.*, 2001) and pilot clinical studies (Gerhardt *et al.*, 2001; Gupta *et al.*, 2001), remains a matter of debate.

Chemotactic agonists, such as platelet-activating factor (PAF), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), or LTB_4, bind to their specific G protein-coupled receptors (GPCR), leading to the activation of MAPK and the mobilisation of Ca^{2+}, which are pivotal signalling molecules that regulate a number of functional processes of PMNL, including chemotaxis, degranulation, formation of reactive oxygen species (ROS), release of arachidonic acid (AA), and LT biosynthesis (Herlaar & Brown, 1999; Belcheva & Coscia, 2002; Johnson & Druey, 2002). Recently, we showed that 11-keto-BAs (AKBA, KBA, in the range of $10-30\,\mu M$) are potent activators of $p42^{MAPK}$ and p38 MAPK, and stimulate Ca^{2+} mobilisation in PMNL (Altmann *et al.*, 2002). Comparison of these actions of BAs with those of chemotactic agonists led us to conclude that BAs may possibly function as ligands of a certain GPCR. In this study, we investigated if BAs are able to induce the functional cellular responses in PMNL and if a $G_{i/o}$-coupled heterotrimeric G protein mediates BA-induced $MAPK/Ca^{2+}$ signalling. Finally, we demonstrate that BAs induce signalling responses depending on the cell type.

Methods

Materials

A-β-BA, β-BA, AKBA, and KBA were purchased from ChromaDex (Laguna Hills, CA, U.S.A.). Nycoprep was from PAA Laboratories (Linz, Austria); diphenyleneiodonium chloride (DPI), phorbol 12-myristate 13-acetate (PMA), Ca^{2+}-ionophore A23187, and fMLP were from Sigma Chemical Co. (Deisenhofen, Germany); BAPTA/AM, LY-294002, SB203580, U0126, and Fura-2/AM were from Calbiochem (Bad Soden, Germany); RO-31-8425 was from Alexis (Grünberg, Germany); 2′,7′-dichlorofluorescein diacetate (DCF-DA) and lucigenin were from Molecular Probes European BV (Leiden, Netherlands); and wortmannin from Biotrend (Colonia, Germany). RPMI-1640 medium was from Gibco-BRL (Grand Island, NY, U.S.A.), and fetal calf serum was obtained from Boehringer Mannheim (Mannheim, Germany). Human TGF-β1 was purified from outdated platelets, as described (Werz *et al.*, 1996). Calcitriol was kindly provided by Dr H. Wiesinger (Schering AG). [^{3}H]AA was from

Biotrend (Colonia, Germany); and high-performance liquid chromatography (HPLC) solvents were from Merck (Darmstadt, Germany).

Cells

Human PMNL were freshly isolated from leucocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation for the preparation of leucocyte concentrates. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes, as described (Werz *et al.*, 2002a). PMNL were finally resuspended in PBS plus $1\,mg\,ml^{-1}$ glucose (PG buffer), or alternatively in PBS plus $1\,mg\,ml^{-1}$ glucose and $1\,mM\,CaCl_2$ (PGC buffer), as indicated.

HL60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, $100\,\mu g\,ml^{-1}$ streptomycin, and $100\,U\,ml^{-1}$ penicillin. For differentiation towards granulocytic cells, cells were seeded at a density of 2×10^5 cells ml^{-1} and cultured in the presence of 1.5% ($v\,v^{-1}$) dimethylsulphoxide, $500\,pM$ calcitriol, and $2\,ng\,ml^{-1}$ TGFβ for 4 days.

Mono Mac (MM) 6 cells were cultured and differentiated with TGFβ and calcitriol, as described (Werz *et al.*, 1996).

Determination of release of [^{3}H]-labelled AA from PMNL

Freshly isolated PMNL were resuspended at 2×10^6 in 1 ml RPMI 1640 medium containing $4.8\,nM$ [^{3}H]AA (corresponding to $0.25\,\mu Ci\,ml^{-1}$, specific activity $200\,Ci\,mmol^{-1}$) and incubated for 120 min at 37°C in 5% CO_2 atmosphere. Thereafter, the cells were collected by centrifugation, washed once with PBS and twice with PBS containing $2\,mg\,ml^{-1}$ fatty acid-free albumin, to remove unincorporated [^{3}H]AA. Labelled PMNL (5×10^6) were resuspended in 1 ml PGC buffer containing $2\,mg\,ml^{-1}$ fatty acid-free albumin. The reaction was started by addition of the indicated stimuli. After 5 min at 37°C, the samples were placed on ice for 2 min and cells were centrifuged at $400\times g$ for 5 min at RT. Aliquots ($100\,\mu l$) of the supernatants were measured in a beta-counter (Micro Beta Trilux, Perkin Elmer, Foster City, CA, U.S.A.) to detect the amounts of [^{3}H]-labelled AA released into the medium.

Determination of cellular peroxide formation

Measurement of peroxides was conducted using the peroxide-sensitive fluorescence dye DCF-DA. Freshly isolated PMNL (1×10^7), HL 60 cells (1×10^7), or MM6 cells (5×10^6) were resuspended in 1 ml PGC buffer and preincubated with DCF-DA ($1\,\mu g\,ml^{-1}$ for PMNL and MM6; $10\,\mu g\,ml^{-1}$ for HL 60 cells) for 2 min at 37°C in a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, U.S.A.) with continuous stirring. The fluorescence emission at 530 nm was measured after excitation at 485 nm. The mean fluorescence data measured 5 min after stimulus addition are given as arbitrary units.

Detection of superoxide anion

Freshly isolated PMNL (2×10^5) were resuspended in 200 μl of 100 mM NaCl, 5 mM KCl, 25 mM NaHCO$_3$, 20 mM HEPES, pH 7.4, and transferred to a 96-well plate. Lucigenin (25 μM, final concentration) and 1 mM CaCl$_2$ were added and cells were stimulated with the indicated stimuli. After stirring, samples were measured in duplicates at 37°C. The chemiluminescence (CL) was recorded using a Micro Luminat Plus LB 96 V (Berthold, Bad Wildbad, Germany) at intervals of 6 s (two cycles) in a total detection time of 3 min. The detected CL was summarised over two intervals and plotted *versus* blank values.

Measurement of intracellular Ca^{2+} levels

The determination of intracellular Ca^{2+} levels was performed as described previously (Werz, 2002a). In brief, freshly isolated PMNL (1×10^7), HL 60 cells (1×10^7 buffer), or MM6 cells (3×10^6) were resuspended in 1 ml PGC buffer and incubated with 2 μM Fura-2/AM for 30 min at 37°C. After washing, cells were finally resuspended in 1 ml PGC buffer and transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, U.S.A.) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz *et al.* (1985), whereas F_{max} (maximal fluorescence) was obtained by lysing the cells with 0.5% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Determination of MAPK activation by SDS–PAGE and Western blotting

Freshly isolated PMNL, HL60, or MM6 cells (5×10^6 each) were resuspended in PGC buffer; the final volume was 100 μl. After addition of the indicated stimuli, samples were incubated at 37°C and the reaction was stopped by addition of 100 μl of ice-cold 2× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample-loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w v^{-1}), 10% β-mercaptoethanol), vortexed and heated for 6 min at 95°C. Total cell lysates (20 μl) were mixed with 4 μl of glycerol/0.1% bromophenolblue (1 : 1, v v^{-1}) and analysed by SDS–PAGE using a Mini Protean system (Bio-Rad, Hercules, CA, U.S.A.) on a 10% gel. After electroblot to nitrocellulose membrane (Amersham Pharmacia, Little Chalfont, U.K.), membranes were blocked with 5% nonfat dry milk in 50 mM Tris/HCl, pH 7.4, and 100 mM NaCl (Tris-buffered saline (TBS)) plus 0.1% Tween 20 for 1 h at RT. Membranes were washed and then incubated with primary antibody (AB) overnight at 4°C. Phospho-specific ABs recognising p44/42^{MAPK} (Thr202/Tyr204) and p38 MAPK (Thr180/Tyr182) were obtained from New England Biolabs (Beverly, MA, U.S.A.), and used as 1 : 2000 dilution. The membranes were washed and incubated with 1 : 1000 dilution of alkaline phosphatase-conjugated immunoglobulin G (Sigma Chemical Co.) for 2 h at RT. After washing with TBS and TBS plus 0.1% NP-40, proteins were visualised with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$).

Determination of 5-LO product formation in PMNL

To determine 5-LO product formation in intact cells, 1×10^7 freshly isolated PMNL were finally resuspended in 1 ml PG buffer. When 5-LO product formation was assayed in the absence of Ca^{2+}, PMNL were finally resuspended in 1 ml PG buffer and 1 mM EDTA and 30 μM BAPTA/AM were added. The reaction was started by simultaneous addition of exogenous AA with the indicated BAs. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol, and 30 μl of 1 N HCl, and 200 ng prostaglandin B$_1$ and 500 μl of PBS were added. Formed AA metabolites were extracted using C-18 solid-phase extraction columns and analysed by HPLC as described (Werz *et al.*, 1997). 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells, which includes LTB$_4$ and its all-*trans* isomers, 5(S),12(S)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(S),12(S)-DiHETE), 5(S)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE), and 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE). 5-HETE and 5-HPETE coelute as one major peak; integration of this peak represents both eicosanoids. Cysteinyl LTs (LTC$_4$, D$_4$, and E$_4$) were not detected and oxidation products of LTB$_4$ were not determined.

Statistics

The results are presented as mean $\pm$ s.e. The program 'GraphPad PRISM 3.0' was used for statistical comparisons. Statistical evaluation of the data was performed using Student's *t*-test for unpaired observations. A *P*-value of < 0.05 was considered significant.

Results

11-Keto-BAs stimulate the release of superoxide anion and ROS in PMNL

In order to explore the effects of BAs on PMNL, we sought to determine whether BAs are capable of eliciting functional processes connected to Ca^{2+} mobilisation and MAPK activation. Phagocytes undergo an oxidative burst in response to different agonists, resulting in the release of ROS *via* the NADPH oxidase. PMNL, preloaded with the ROS-sensitive dye DCF-DA, were stimulated with BAs (30 μM, each) and for comparison with 1 μM fMLP, 2.5 μM ionophore A23187, and 100 nM PMA. After 5 min, ROS formation was determined by analysing the fluorescence of the oxidised dye. In agreement with the literature (Roos *et al.*, 1976; Simchowitz & Spilberg, 1979), fMLP, ionophore A23187, or PMA caused a rapid formation of ROS in PMNL (Figure 1). AKBA and KBA strongly upregulated the formation of ROS, whereas β-BA and A-β-BA had only moderate effects (Figure 1). Notably, the magnitude of AKBA- (or KBA-) induced ROS formation was much more pronounced as compared to fMLP, being in a close range of the efficacy of ionophore and PMA. Similar results were obtained when the formation of superoxide anion (O$_2^-$) was determined by measuring the chemiluminescence of the metabolised lucigenin. Thus, stimulation of PMNL with 30 μM AKBA resulted in a 12-fold increase in O$_2^-$ formation compared to unstimulated cells, whereas fMLP (1 μM) and
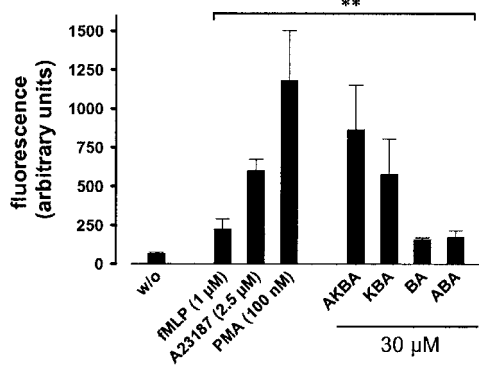
Figure 1 11-Keto-BAs induce the generation of ROS in PMNL. Freshly isolated PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with DCF-DA ($1 \mu g\,ml^{-1}$) for 2 min at 37°C, prior addition of the indicated stimuli. The generation of peroxides was measured as described. Data determined 5 min after addition of stimuli are expressed as the mean of the fluorescence given in arbitrary units $\pm$ s.e., $n = 4$. Student's t-test; **$P < 0.01$.

PMA (100 nM) caused 4- and 20-fold elevations, respectively (not shown).

AKBA-induced ROS formation involves NADPH oxidase, PI 3-K, and $p42/44^{MAPK}$, and requires Ca^{2+}

To further dissect the signalling pathways leading to ROS formation, pharmacological inhibitors of potential signalling molecules were examined using the DCF-DA fluorescence assay. PMA, used as a control, binds to PKC isoenzymes, stimulating NADPH oxidase by multiple phosphorylations of $p47^{phox}$ (Heyworth & Badwey, 1990). DPI, a direct inhibitor of NADPH oxidase (Hancock & Jones, 1987), almost completely abolished PMA- as well as AKBA-induced ROS formation (Figure 2a). Also, chelation of Ca^{2+} by $30 \mu M$ BAPTA/AM and 1 mM EDTA clearly reduced AKBA- or PMA-induced ROS formation (Figure 2a). As shown in Figure 2b, PMA-induced ROS formation was only slightly affected by wortmannin (50 nM) or LY-294002 ($30 \mu M$), inhibitors of phosphatidylinositol 3-kinase (PI 3-K), or by the p38 MAPK inhibitor SB203580 ($10 \mu M$). However, U0126 ($3 \mu M$) that blocks the activation of $p42/44^{MAPK}$ partially reduced ROS generation and the PKC inhibitor RO-318425 ($1 \mu M$) totally abolished the effects of PMA. In contrast, AKBA-induced ROS formation was not at all affected by PKC inhibition (RO-318425), but was clearly reduced by wortmannin or LY-294002, and by U0126, implying an involvement of PI 3-K and $p42/44^{MAPK}$, respectively (Figure 2b). Also, for AKBA-induced ROS formation, a role for p38 MAPK is not apparent.

11-Keto-boswellic acids cause the release of [³H]AA from PMNL

In PMNL, AA is released from phospholipids upon cell stimulation by the cytosolic PLA_2 ($cPLA_2$). Since $cPLA_2$ is activated by phosphorylation at serine residues by members of the MAPK family and/or elevation of the intracellular Ca^{2+} levels (Gijon & Leslie, 1999), BAs were tested for their ability to elevate the liberation of AA from PMNL. AKBA and KBA considerably enhanced the release of [³H]-labelled AA, whereas
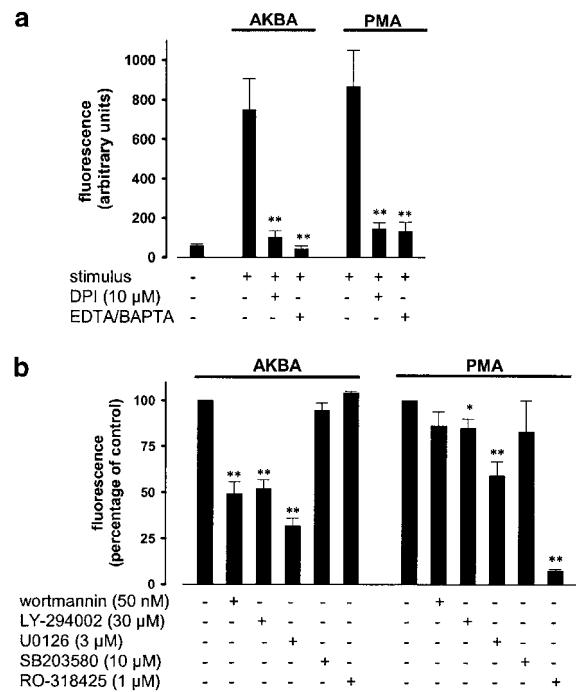

Figure 2 AKBA-induced ROS formation involves NADPH oxidase, PI 3-K, and $p42/44^{MAPK}$, and requires Ca^{2+}. PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with the indicated compounds for 20 min at RT, prior addition of DCF-DA ($1 \mu g\,ml^{-1}$). After another 2 min, AKBA ($30 \mu M$) or PMA (100 nM) were added and the generation of peroxides was measured as described above. (a) Effects of DPI and Ca^{2+} depletion. Data determined 5 min after addition of stimuli are expressed as the mean of the fluorescence given in arbitrary units $\pm$ s.e., $n = 3$. Student's t-test; **$P < 0.01$. (b) Effects of pharmacological protein kinase inhibitors. Data were determined 5 min after addition of stimuli and expressed as the percentage of the mean fluorescence $\pm$ s.e., $n = 4$, of control. Student's t-test; *$P < 0.05$, **$P < 0.01$. The control values (100%) in the absence of inhibitors for cells stimulated with AKBA were 425 ± 57, and for PMA 675 ± 103 arbitrary fluorescence units.

β-BA and A-β-BA showed only weak effects (Figure 3). Also, under these experimental settings, AKBA and KBA caused even more pronounced responses as compared to $1 \mu M$ fMLP. The fMLP- as well as the AKBA-induced AA release was completely abolished when Ca^{2+} was removed by chelation using EDTA and BAPTA/AM (not shown). Experiments using SB203580 ($10 \mu M$) and U0126 ($3 \mu M$), in order to inhibit p38 MAPK and $p42/44^{MAPK}$ activities, respectively, were performed to estimate the importance of these MAPK for AA liberation. The effect of AKBA was partially suppressed by U0126 ($53 \pm 4.6\%$), but was unaffected by SB203580 (not shown), implying that $p42/44^{MAPK}$, but apparently not p38 MAPK, may contribute to the AKBA-induced AA release.

Effects of 11-keto-boswellic acids on cellular 5-LO product formation in PMNL

In intact cells, 5-LO can be activated upon elevation of the intracellular Ca^{2+} levels and/or phosphorylation by p38 MAPK-regulated MAPKAPKs and $p42/44^{MAPK}$ (Werz et al., 2000; 2002a, b). Although 11-keto BAs are direct inhibitors of 5-LO (Safayhi et al., 1992; 1995), we speculated that, due to their potential to mobilise Ca^{2+} and to activate MAPK, BAs could also stimulate 5-LO in intact cells. As shown in

Figure 4a, when 11-keto-BAs were added to PMNL together with 20 μM AA, AKBA and KBA (30 μM each) caused 3.8- and 2.4-fold increases in 5-LO product formation *versus* control cells that had been stimulated with AA alone, whereas no upregulatory effects were observed for β-BA and A-β-BA. Notably, such upregulatory effects of the 11-keto-BAs were most prominent when Ca^{2+} was chelated by EDTA and BAPTA/AM, whereas, in the presence of Ca^{2+}, AKBA increased 5-LO product formation only by 1.9-fold. Expanding the preincubation period with BAs prior addition of AA (>1 min) strongly reduced the effects of BAs (not shown). The dose–response curve shown in Figure 4b reveals that, at higher concentrations (100 μM), AKBA fails to stimulate 5-LO, possibly due to direct inhibitory enzyme interaction. In order to determine if p38 MAPK and/or p42/44^{MAPK} are required for AKBA-induced 5-LO activation, PMNL were preincubated with 3 μM U0126 and/or 10 μM SB203580 prior stimulation. Upregulation of 5-LO product formation by AKBA (in the absence of Ca^{2+}) was not significantly suppressed by these

inhibitors (not shown), suggesting that MAPK are not determinants for AKBA-induced 5-LO activation.

Prolonged exposure of 5-LO to elevated Ca^{2+} or oxidants such as ROS leads to a rapid inactivation of 5-LO (Ford-Hutchinson *et al.*, 1994). Hence, it appeared possible, that the potent 5-LO inhibition by AKBA in intact cells after longer preincubation periods (30 min) prior cell stimulation is related to the prominent Ca^{2+} mobilisation and the accompanied
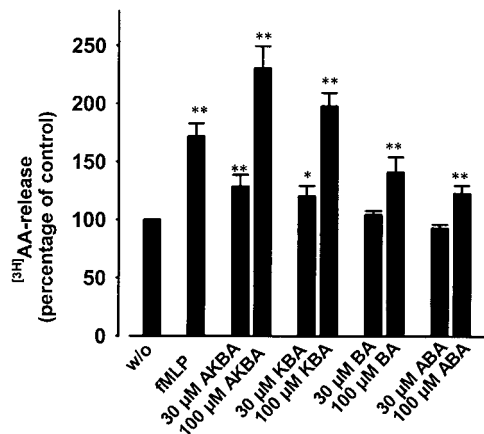

Figure 3 11-Keto-BAs elicit the liberation of AA in PMNL. Freshly isolated PMNL (2×10^6 in 1 ml RPMI 1640 medium) were prelabelled with 0.25 μCi ml^{-1} [^{3}H]AA for 120 min at 37°C and 5% CO_2. After unincorporated [^{3}H]AA was removed, cells (5×10^6 in 1 ml PGC buffer, containing 2 mg ml^{-1} fatty acid-free albumin) were treated with the indicated additives and incubated for 5 min at 37°C. Free (nonesterified) [^{3}H]AA was determined as described in Methods. Results are given as mean$\pm$s.e. ($n=4$). Student's *t*-test; *$P<0.05$; **$P<0.01$.

Figure 4 Effects of 11-keto-BAs on the formation of 5-LO metabolites. (a) Freshly isolated PMNL (5×10^6) were resuspended in 1 ml PG buffer containing 1 mM EDTA and preincubated with 30 μM BAPTA/AM for 15 min. Then, cells were stimulated with 20 μM AA alone or together with the indicated BAs (30 μM, each). After 10 min at 37°C, 5-LO products were determined by HPLC. Results are given as mean + s.e., $n=4$–6. Student's *t*-test; **$P<0.01$. (b) Dose–response curve of AKBA. PMNL (5×10^6) were resuspended in 1 ml PG buffer containing 1 mM EDTA and preincubated with 30 μM BAPTA/AM for 15 min. Then, cells were stimulated with 20 μM AA alone or together with the indicated amounts of AKBA. After 10 min at 37°C, 5-LO products were determined. Results are given as mean + s.e., $n=4$–5. (c) Inhibition of 5-LO. PMNL (5×10^6) were resuspended in either 1 ml PGC buffer or PG buffer containing 1 mM EDTA and 30 μM BAPTA/AM. After 15 min at 37°C, AKBA was added, and cells were incubated for another 30 min at 37°C. Then, CaCl$_2$ was adjusted to 1 mM in all incubations and cells were immediately stimulated with 2.5 μM ionophore and 40 μM AA. After 10 min, 5-LO products were determined. Results are given as mean + s.e., $n=3$.
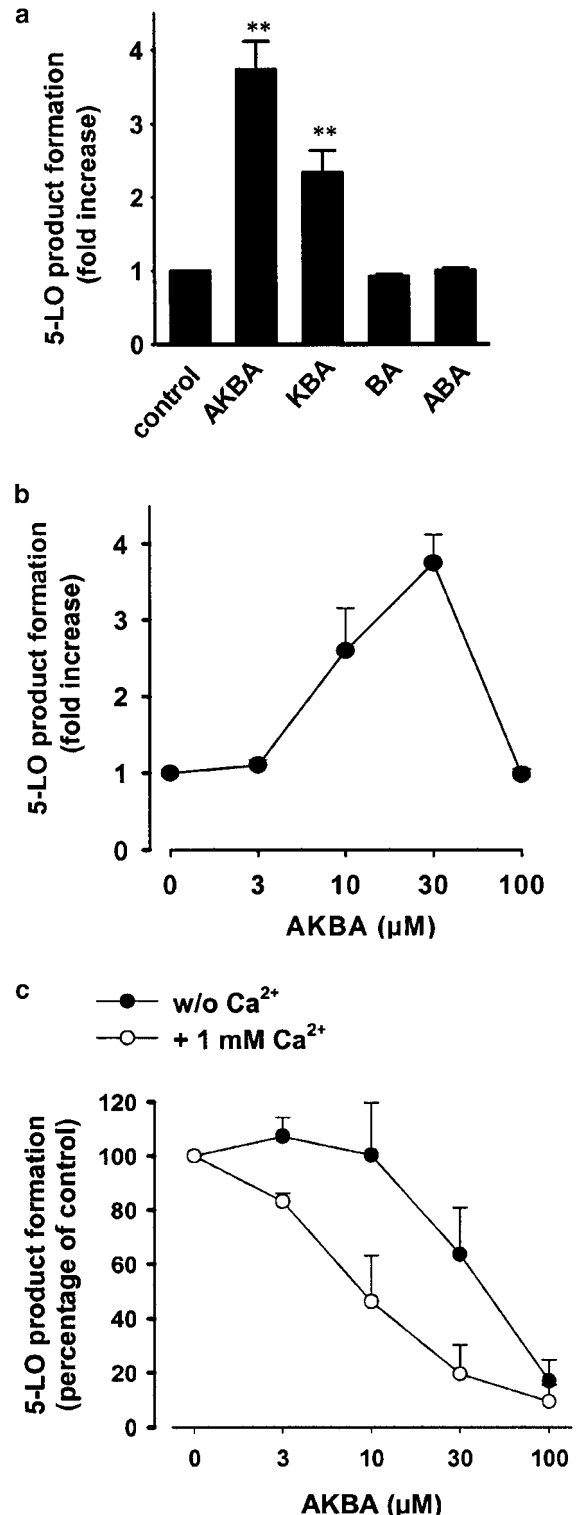
ROS release. PMNL were preincubated for 30 min with AKBA in the presence of Ca^{2+}, which results in Ca^{2+} mobilisation and ROS formation. Alternatively, Ca^{2+} was removed by 1 mM EDTA and 30 μM BAPTA/AM during this preincubation period, conditions where ROS release does not occur. Subsequently, after preincubation, all samples were adjusted to 1 mM Ca^{2+} and cells were immediately stimulated with ionophore and AA to induce 5-LO product formation in intact cells. As can be seen from Figure 4c, the efficacy of AKBA to inhibit 5-LO product formation was strongly impaired when cells were preincubated in the absence of Ca^{2+} (IC_{50} approx. 50 μM), as compared to conditions when Ca^{2+} was present (IC_{50} approx. 8 μM). Thus, potent 5-LO inhibitory effects of AKBA in intact cells by may require Ca^{2+}-mediated processes, such as ROS formation.

Pertussis toxin (PTX) attenuates boswellic acid-induced mobilisation of Ca^{2+} and MAPK activation in PMNL

The proximal signalling pathways involved, as well as the kinetics for Ca^{2+} mobilisation and MAPK activation, resembled those of chemotactic factors, such as fMLP, that act *via* G protein-coupled receptors (GPCR). In order to determine a possible role of G_i or G_0 proteins in the AKBA-induced Ca^{2+} release and MAPK activation, the effects of PTX, an irreversible inhibitor of $G\alpha_{i/0}$ subunits of hetero-trimeric G proteins, were assessed in human isolated PMNL. As shown in Figure 5a, PTX (2 μg ml^{-1}) suppressed the Ca^{2+} response induced by 30 μM AKBA in a similar fashion as compared to fMLP stimulation. Similarly, PTX clearly reduced the activation of p42^{MAPK} in PMNL, stimulated with AKBA or KBA, whereas activation of p38 MAPK was only moderately reduced (Figure 5b). In control experiments (Table1 and Figure 5b), PTX also partially decreased the fMLP-induced activation of MAPK and Ca^{2+} mobilisation, whereas MAPK activation (not shown) and Ca^{2+} mobilisation (Table 1) induced by ionomycin (that circumvents G protein signalling) were not suppressed by PTX.

Moreover, PTX (2 μg ml^{-1}) partially inhibited AKBA-induced ROS formation ($21.1 \pm 7.6\%$), but did not at all affect ROS production in PMNL stimulated by PMA. On the other hand, PTX caused only marginal suppression ($8.3 \pm 4.1\%$) of AKBA-induced 5-LO activity in PMNL.

Cell type-dependent induction of MAPK activation, Ca^{2+} mobilisation and ROS generation by BAs

The effects of BAs on the mobilisation of Ca^{2+}, activation of MAPK and formation of ROS were further investigated in various haematopoietic cell lines. For monocytic MM6 cells differentiated with calcitriol and TGFβ, none of the four different BAs (up to 100 μM) induced Ca^{2+} mobilisation or activation of MAPK, and also no significant induction of ROS formation was observed (not shown). In control experiments, PAF, LTB$_4$ or fMLP caused pronounced elevation of intracellular Ca^{2+}, demonstrating that G protein-coupled Ca^{2+} mobilisation is operative in these cells. Similarly, BAs also failed to activate the B-lymphocytic cell line BL41-E-95-A in this respect (not shown).

In contrast, the effects of BAs in differentiated granulocytic HL60 cells resembled those observed in PMNL from human blood. Thus, AKBA considerably induced mobilisation of
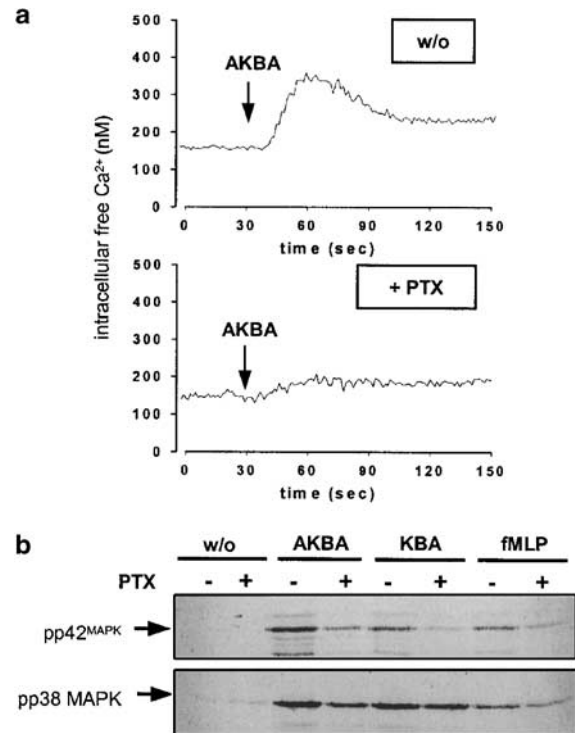
Figure 5 PTX suppresses Ca^{2+} mobilisation and activation of MAPK in PMNL induced by boswellic acids. (a) Ca^{2+} mobilisation. Freshly isolated PMNL (1 ± 10^7 ml^{-1} PGC buffer) were loaded with 2 μM Fura-2 for 30 min at 37°C. Then, cells were preincubated with or without 2 μg ml^{-1} PTX for 60 min at 37°C. After addition of 30 μM AKBA, the fluorescence was measured and intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment out of four. (b) MAPK activation. Freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were preincubated with or without 2 μg ml^{-1} PTX for 60 min at 37°C and then stimulated with 30 μM AKBA, 30 μM KBA, 1 μM fMLP, or left untreated. After 1.5 min at 37°C, incubations were terminated by addition of the same volume of ice-cold SDS-b. Samples were electrophoresed and analysed for dually phosphorylated p38 MAPK or p44/42^{MAPK} by Western blotting.

Table 1 Effects of PTX on Ca^{2+} mobilisation

Stimulus	Ca^{2+} mobilisation (percentage of control)
Ionomycin (2.5 μM)	94.1 ± 8.3
fMLP (100 nM)	39.6 ± 9.7
AKBA (30 μM)	43.8 ± 16.8

Freshly isolated PMNL (1×10^7 ml^{-1} PGC buffer) were loaded with 2 μM Fura-2 for 30 min at 37°C. Then, cells were preincubated with or without 2 μg ml^{-1} PTX for 60 min at 37°C. After addition of the indicated stimuli, the fluorescence was measured and intracellular free Ca^{2+} was calculated as described. Values (mean $\pm$ s.e., $n = 4$) are given as the percentage of Ca^{2+} mobilisation of PTX-treated cells *versus* control cells that received no PTX.

Ca^{2+}, stimulated activation of p38 MAPK and p42^{MAPK}, and upregulated the formation of ROS (Figure 6). Interestingly, KBA was less effective, whereas β-BA and A-β-BA also stimulated HL60 cells for MAPK activation and release of ROS. Nevertheless, β-BA and A-β-BA failed to substantially mobilise Ca^{2+} in differentiated HL60 cells. It should be noted
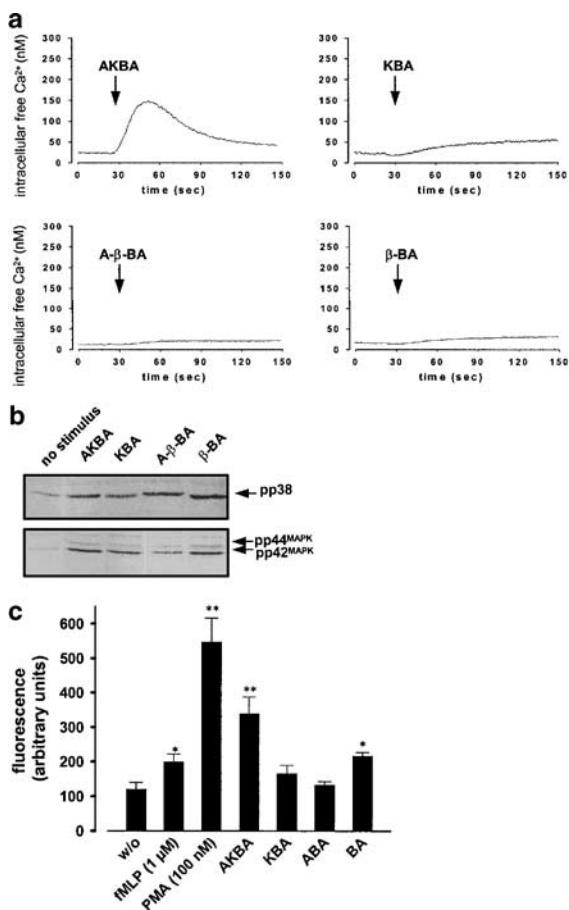
Figure 6 Effects of BAs on Ca^{2+} release, MAPK activation, and ROS production in granulocytic HL60 cells. HL60 cells were differentiated towards granulocytic cells in the presence of 1.5% DMSO, $2\,ng\,ml^{-1}$ TGFβ, and 500 pM calcitriol for 4 days. (a) Mobilisation of Ca^{2+}. Cells ($10^7\,ml^{-1}$ PGC buffer) were loaded with $2\,\mu M$ Fura-2 for 30 min at $37°C$. After addition of BAs ($50\,\mu M$, each) or $1\,\mu M$ fMLP, the fluorescence was measured and intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment out of 4–5, respectively. (b) MAPK activation. Cells ($5 \times 106/100\,\mu l$ PGC buffer) were stimulated with $30\,\mu M$ of BAs, $1\,\mu M$ fMLP, or left untreated. After 1.5 min at $37°C$, incubations were terminated by addition of the same volume of ice-cold SDS-b. Samples were electrophoresed and analysed for dually phosphorylated p38 MAPK or p44/42^{MAPK} by Western blotting. (c) ROS formation. Cells ($5 \times 10^7\,ml^{-1}$ PGC buffer) were preincubated with DCF-DA ($10\,\mu g\,ml^{-1}$) for 2 min at $37°C$ prior addition of the indicated stimuli. The generation of peroxides was measured as described. Data determined 5 min after addition of stimuli are expressed as the mean of the fluorescence given in arbitrary units $\pm$ s.e., $n=4$. Student's *t*-test; $*P<0.05$; $**P<0.01$.

that undifferentiated HL60 cells did neither respond to AKBA nor to the other BAs (not shown), implying that a certain signalling component(s), induced during differentiation, is required to transduce the effects of BAs.

Discussion

We have recently reported that 11-keto-BAs potently stimulate the elevation of intracellular Ca^{2+} levels and activate p38 MAPK as well as p42^{MAPK} (Altmann *et al.*, 2002), which are pivotal signalling events that regulate numerous effectors of

PMNL. In the present study, we show that 11-keto-BAs in fact elicit functional responses of PMNL or granulocytic HL60 cells such as ROS generation, increased liberation of AA, and its subsequent metabolism by 5-LO. Investigation of the signalling molecules involved and comparison of the kinetics with those of chemotactic ligands for leukocytes, that is, fMLP, PAF or LTB$_4$, led us to conclude that 11-keto-BAs may transduce their signals in a common way as chemotactic ligands that involve GPCR signalling (see Altmann *et al.* (2002) and references therein). Since elevated levels of AA and ROS are critical inducers of caspase-mediated apoptosis (Hampton *et al.*, 1998; Taketo & Sonoshita, 2002), our findings may also provide a molecular basis for the 11-keto-BA-induced caspase activation (Liu *et al.*, 2002a, b) and the apoptotic effects observed in various cancer cell lines (Shao *et al.*, 1998; Glaser *et al.*, 1999; Hoernlein *et al.*, 1999; Jing *et al.*, 1999; Liu *et al.*, 2002a, b).

O_2^- is the precursor of ROS, which are essential for the host defence against microorganisms. The NADPH oxidase of leucocytes is the major source of O_2^- released upon agonist challenge (Chanock *et al.*, 1994). Activation of NADPH oxidase requires the presence of Ca^{2+} and multiple phosphorylations of the subunit p47^{phox} by certain PKC isoenzymes or other kinases (Heyworth & Badwey, 1990; Chanock *et al.*, 1994; Dewas *et al.*, 2000). We demonstrate that, according to their ability to stimulate Ca^{2+} mobilisation and MAPK activation (compare Altmann *et al.*, 2002), 11-keto-BAs, but not BAs lacking the 11-keto group, induced a rapid and robust formation of O_2^- and of ROS in PMNL, as determined by lucigenin chemiluminescence and the oxidation of DCF-DA, respectively. Since the NADPH oxidase inhibitor DPI (Hancock & Jones, 1987) abolished ROS formation, ROS derived from the NADPH oxidase system may indeed be responsible. Pharmacological targeting of the proximal signalling pathways revealed that AKBA-induced ROS formation seemingly depends on PI 3-K and on the p42/44^{MAPK} pathway, but does not require p38 MAPK or PKC. In this context, it is interesting that the fMLP-induced phosphorylation of the p47^{phox} component in neutrophils was suppressed by inhibition of p42^{MAPK} (Dewas *et al.*, 2000) and PI 3-K (Ding *et al.*, 1995), but not when p38 MAPK was blocked by SB203580 (Dewas *et al.*, 2000). In contrast, our control experiments utilising PMA as a direct activator of PKC confirm the findings by others, showing that PKC, and to a minor extent also p42^{MAPK}, are required for NADPH oxidase-dependent ROS formation (Cox *et al.*, 1985; Heyworth & Badwey, 1990; Dewas *et al.*, 2000), whereas a role of PI 3-K or p38 MAPK is not readily apparent. Notably, Ca^{2+} signalling pathways are determinants for ROS formation, since chelation of Ca^{2+} strongly suppressed the signals induced by AKBA and also by PMA. Together, 11-keto BAs may stimulate ROS formation by mobilisation of Ca^{2+} and by activation of p42/44^{MAPK}, apparently involving PI 3-K.

The release of free AA by leukocytes is an important step in the onset of inflammatory reactions and the cPLA$_2$, which is regulated by Ca^{2+} and phosphorylation by MAPK, appears to play a major role for AA liberation in PMNL (Gijon & Leslie, 1999). 11-keto BAs, but not β-BA and A-β-BA, induced the release of considerable amounts of AA, with a similar efficacy as fMLP. The doses of the respective BAs were somewhat higher than those needed for the formation of ROS, which might be due to the fact that cPLA$_2$ favours a sustained Ca^{2+} influx (Qiu *et al.*, 1998), which in turn requires higher

concentrations of AKBA or KBA (unpublished observations). In agreement with findings reported for ligands acting *via* GPCR (Qiu *et al.*, 1998), AKBA-induced AA liberation also depended on Ca^{2+} and p42/44^{MAPK}.

Although BAs have been initially reported as direct-type inhibitors of the 5-LO enzyme, without reducing or iron-chelating properties (Safayhi *et al.*, 1992; 1995), we demonstrate that AKBA can paradoxically stimulate cellular 5-LO, when added to PMNL concomitantly with AA. Also, it was reported that low concentrations of *B. serrata* extracts enhanced ionophore-stimulated 5-LO product synthesis in PMNL (Safayhi *et al.*, 2000), and 3-oxo-tirucallic acid, that acts as a direct 5-LO inhibitor in cell-free systems, induced and upregulated 5-LO activity in human neutrophils (Boden *et al.*, 2001). In intact cells, mobilisation of Ca^{2+}, and also phosphorylation by MAPKAPKs and by p42/44^{MAPK}, activates 5-LO for product formation (Werz *et al.*, 2000; 2002a, b). Interestingly, AKBA-induced 5-LO activation was most prominent in the absence of Ca^{2+}, and was not sensitive to MAPK inhibitors and hardly sensitive to PTX. Thus, additional unknown factors or pathways induced by AKBA seem operative, which still remain to be determined.

A discrepancy in the efficacy of AKBA is evident for suppression of 5-LO in intact cells (IC$_{50} \approx 2$–$5 \mu M$) and cell-free systems (IC$_{50} \approx 15$–$50 \mu M$) (Safayhi *et al.*, 1995; Werz *et al.*, 1997; 1998). Thus, in intact cells, additional factors are operative for the suppression of 5-LO. Since prolonged exposure to elevated Ca^{2+} or oxidants rapidly inactivates 5-LO (Ford-Hutchinson *et al.*, 1994), it appeared possible that the potent 5-LO inhibition by AKBA in intact cells is related to the prominent ROS release occurring during preincubation periods (15–30 min) prior cell stimulation. In fact, Ca^{2+} depletion, that prevents ROS formation, impaired the efficacy of AKBA in intact cells (Figure 4c), and the respective IC$_{50}$ value fits well with those obtained in cell-free systems (Werz *et al.*, 1998). Intriguingly, the efficacy of AKBA for suppression of 5-LO in MM6 cells (IC$_{50} \approx 15 \mu M$), that are unable to mobilise Ca^{2+} and to produce ROS in response to AKBA, is significantly reduced as compared to PMNL (IC$_{50} \approx 2$–$5 \mu M$).

Upon ligation of their specific GPCR, chemoattractants elicit various functional responses of different leucocytes involving Ca^{2+} mobilisation and activation of MAPK (Herlaar & Brown, 1999; Belcheva & Coscia, 2002). The putative BA receptor, operative in PMNL and mature HL60 cells, seems to be induced during differentiation towards granulocytic cells, since undifferentiated HL60 cells did not respond to BAs with Ca^{2+} mobilisation, MAPK activation or ROS formation. Along these lines, it was found that, in HL60 cells, the G protein-coupled fMLP receptor is also first induced after differentiation (Perez *et al.*, 1992). Nevertheless, differentiated monocytic MM6 cells apparently do not possess any BA-inducible signalling pathways or BA receptor(s), although these cells respond to diverse ligands of GPCR (fMLP, LTB$_4$ or PAF), implying intact G protein signalling pathways in

differentiated MM6 cells. Intriguingly, differentiated MM6 cells also failed to mobilise Ca^{2+} in response to 5(S)-HETE or 5-oxo-ETE, which on the other side caused significant Ca^{2+} mobilisation in PMNL (unpublished observations). Accordingly, even the expression of the putative G protein coupled 5-oxo-ETE receptor was found to be cell type-dependent (O'Flaherty *et al.*, 2000). Thus, we conclude that the expression of the target (–receptor) of AKBA may be restricted to certain cell types. Finally, it should be noted that, in contrast to PMNL, in differentiated HL60 cells also β-BA and A-β-BA caused cell stimulation (Figure 6a and b), suggesting that, at least in HL60 cells, putative receptor subtypes (not present in PMNL) may exist that accept BAs lacking the 11-keto group.

Our experiments using PTX suggest that a $G_{i/o}$-subunit of a heterotrimeric G protein mediates the effects of 11-keto-BAs in PMNL. Remarkably, the activation of p38 MAPK and of 5-LO was less affected by PTX, implying that, for example, PTX-insensitive G_q subunits, as in the case of the PAF receptor (Shimizu *et al.*, 1996), are involved. Also, the PI 3-K inhibitor wortmannin differentially suppressed the activation of p38 MAPK and p42^{MAPK} induced by AKBA (Altmann *et al.*, 2002). It is yet unclear whether 11-keto-BAs act *via* a GPCR or alternatively interfere directly with a G protein. Notably, modulation of G proteins by low molecular weight compounds in the absence of a GPCR, both in a positive or a negative way, has been described (see Breitweg-Lehmann *et al.* (2002) and references therein).

The data presented here imply that BAs, at least in concentrations $\geqslant 10 \mu M$, are potent immunocompetent agents that might be regarded as proinflammatory stimuli. By contrast, cellular and pilot clinical studies indicate the anti-inflammatory effects of *B. serrata* extracts and BAs (Gupta *et al.*, 1992; 2001; Gerhardt *et al.*, 2001; Krieglstein *et al.*, 2001). It is conceivable that, at low concentrations, BAs may have antagonistic activity within certain signalling pathways induced by a second stimulus. In fact, at low concentrations, AKBA (2–8 μM) inhibited the activation of p42/44^{MAPK} in meningioma cells stimulated with platelet-derived growth factor (Park *et al.*, 2002), and, in our hands, BAs (0.3–1 μM) significantly suppressed the PAF-induced Ca^{2+} mobilisation in platelets (unpublished results). Further studies are required to identify the receptor(s) of BAs and the defined mechanisms leading to Ca^{2+} and MAPK signalling, and to reveal if BAs can act as partial agonists at receptors relevant for inflammatory processes. Such knowledge may help to unravel the molecular mechanisms of the anti-inflammatory actions of BAs, and may provide new concepts for the pharmacological intervention with inflammatory diseases.

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