3-*O*-Acetyl-11-keto-boswellic acid decreases basal intracellular Ca^{2+} levels and inhibits agonist-induced Ca^{2+} mobilisation and MAP kinase activation in human monocytic cells*

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Running title: Effects of boswellic acids in monocytic cells

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Abbreviations used: AB, antibodies; AβBA, 3-*O*-acetyl-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-boswellic acid; 2-APB, 2-aminoethoxydiphenylborate; BA, boswellic acid; ERK, extracellular signal-regulated kinase; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GPCR, G protein-coupled receptor; IP_3, inositol-1,4,5-trisphosphate; KBA, 11-keto-boswellic acid; m-3M3FBS; 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide; MAPK, mitogen-activated protein kinase; NCX, Na^+-Ca^{2+}-exchanger; NSCC, nonselective cation channel; PAF, platelet-activating factor; PG buffer, PBS plus 1 mg/ml glucose; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$; PMNL, polymorphonuclear leukocytes; PLC, phospholipase C; RT, room temperature; SDS-b, 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer; SOCC, store-operated Ca^{2+} channel; TG, thapsigargin.

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Abstract

Previously, we showed that 11-keto-boswellic acid (KBA) and 3-*O*-acetyl-11-keto-BA (AKBA) stimulate Ca^{2+} mobilisation and activate mitogen-activated protein kinases (MAPK) in human polymorphonuclear leukocytes (PMNL). Here we addressed the effects of BAs on the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and on the activation of $p38^{MAPK}$ and extracellular signal-regulated kinase (ERK) in the human monocytic cell line Mono Mac (MM) 6. In contrast to PMNL, AKBA concentration-dependently (1 - 30 µM) decreased the basal $[Ca^{2+}]_i$ in resting MM6 cells but also in cells where $[Ca^{2+}]_i$ had been elevated by stimulation with platelet-activating factor (PAF). AKBA also strongly suppressed the subsequent elevation of $[Ca^{2+}]_i$ induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP), PAF or by the direct phospholipase C activator *m*-3M3FBS, but AKBA failed to prevent Ca^{2+} signals induced by thapsigargin or ionomycin. Suppression of Ca^{2+} homeostasis by AKBA was also observed in primary monocytes, isolated from human blood. Moreover, AKBA inhibited the activation of $p38^{MAPK}$ and ERKs in fMLP-stimulated MM6 cells. Although the effects of AKBA could be mimicked by the putative PLC inhibitor U-73122, AKBA appears to operate independent of PLC activity, since the release of intracellular inositol-1,4,5-trisphophate evoked by *m*-3M3FBS was hardly diminished by AKBA. Inhibitor studies indicate that AKBA may decrease $[Ca^{2+}]_i$ by blocking store-operated Ca^{2+} and/or nonselective cation channels. Together, AKBA interferes with pivotal signalling events in monocytic cells that are usually required for monocyte activation by proinflammatory stimuli. Interruption of these events may represent a possible mechanism underlying the reported anti-inflammatory properties of AKBA.

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Introduction

Boswellia serrata (B.S.) gum resin extracts have been traditionally applied in folk medicine for centuries to treat various chronic inflammatory diseases, and experimental data from animal models and clinical studies on humans confirmed an anti-inflammatory potential of B.S. extracts (for review, see Safayhi and Sailer, 1997; Ammon, 2002). Detailed analysis of the ingredients of these extracts revealed that the pentacyclic triterpenes boswellic acids (BAs) possess pharmacological activities and may be responsible for the respective anti-inflammatory properties (Safayhi and Sailer, 1997). Approaches in order to elucidate the cellular and molecular mechanisms underlying the clinical effects of BAs identified 5-lipoxygenase (5-LO) (Safayhi et al., 1992), human leukocyte elastase (Safayhi et al., 1997), toposiomerase I and II (Syrovets et al., 2000)], as well as IκB kinases (Syrovets et al., 2005) as molecular targets. Accordingly, it is speculated that BAs may exert their anti-inflammatory effect mainly by inhibiting the release of proinflammatory leukotrienes from leukocytes (Safayhi et al., 1992) and/or by inhibition of NF-κB and subsequent down-regulation of TNF-alpha expression in activated monocytes (Syrovets et al., 2005).

Stimulation of inflammatory cells by an adequate agonist may evoke a number of functional responses including chemotaxis, phagocytosis, degranulation, formation of reactive oxygen species, release of cytokines and chemokines, and liberation of lipid mediators. The transduction and mediation of such agonist-induced responses requires appropriate intracellular signalling systems that operate at multiple levels. Elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is one central signalling event for cell activation (Li et al., 2002), being involved in the regulation of functional responses such as degranulation or the generation of reactive oxygen species in agonist-challenged leukocytes (Bernardo et al., 1988). Extracellular stimuli, including the platelet-activating factor (PAF) or N-formyl-methionyl-leucyl-phenylalanine (fMLP), increase the $[Ca^{2+}]_i$ in monocytes/macrophages,

which is composed of a rapid release of Ca^{2+} from intracellular stores and a Ca^{2+} influx through plasma membrane Ca^{2+} channels (Randriamampita and Trautmann, 1989). Besides Ca^{2+}, protein phosphorylation is a common signal transduction mechanism integrating extracellular inflammatory signals into leukocyte functions, and in particular MAPK pathways have been shown to play important roles in this respect (Herlaar and Brown, 1999; Johnson and Druey, 2002). Accordingly, inhibitors of these kinases have been developed in order to intervene with inflammatory disorders.

We have recently shown that 11-keto-BAs can activate MAPK and induce Ca^{2+} mobilisation in human isolated polymorphonuclear leukocytes (PMNL) and granulocytic HL-60 cells which could be linked to various functional responses, including release of arachidonic acid, increased formation of LTs and generation of reactive oxygen species (Altmann et al., 2002; Altmann et al., 2004). Since monocytes play key roles in the course of inflammatory processes, we examined the effect of BAs on the Ca^{2+} homeostasis and MAPK pathways in human monocytic Mono Mac (MM) 6 cells. Interestingly, in contrast to PMNL or HL-60 cells, AKBA exerted opposite effects in MM6 cells, inasmuch as it (I) decreased basal $[Ca^{2+}]_i$, (II) inhibited agonist-induced Ca^{2+} mobilisation, and (III) blocked agonist-induced activation of $p38^{MAPK}$ and ERKs. These findings support an anti-inflammatory potential of AKBA.

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Materials and Methods

Materials

BAs were prepared as described previously (Jauch and Bergmann, 2003). 2,4,6-Trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS) was a generous gift from Dr. TG Lee, SIGMOL (Korea). U-73122 and SK&F96365 were purchased from Calbiochem (Bad Soden, Germany); Fura-2/AM was from Alexis (Grünberg, Germany); PAF was from Cayman Chemicals (Ann Arbor, MI); 2-aminoethoxydiphenylborate (2-APB), Tocris (Bristol, UK); all other chemicals were obtained from Sigma (Deisenhofen, Germany).

Cells

MM6 cells were maintained in RPMI 1640 medium with glutamine supplemented with 10 % fetal calf serum, 100 µg/ml streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, 1 × nonessential amino acids, 1 mM oxalacetic acid and 10 µg/ml bovine insulin. All cultures were seeded at a density of 2×10^5 cells/ml. MM6 cells were treated with 2 ng/ml transforming growth factor β and 50 nM calcitriol for 4 days. Cells were harvested by centrifugation (200 × g, 10 min at RT) and washed once in phosphate-buffered saline pH 7.4 (PBS). To exclude toxic effects of BAs during various incubation periods, the viability of MM6 cells was analysed by trypan blue exclusion. Incubation with 30 µM AKBA or 3 µM U-73122 at 37°C for up to 30 min caused no significant change in the number of viable cells.

Human PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany) as described (Werz et al., 2002). In brief, venous blood was taken from healthy adult donors and leukocyte concentrates were prepared by centrifugation at 4000 × g/20 min/20°C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes. Monocytes were obtained from the same leukocyte concentrates after dextran

sedimentation and centrifugation on Nycoprep cushions. The mononuclear cells including lymphocytes and monocytes appear as a layer on Nycoprep cushion after centrifugation. The cells were washed twice with PBS pH 5.9 containing 2 mM EDTA, resuspended in RPMI-1640 supplemented with 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 20 % human plasma, and spread in cell culture flasks at 37°C and 5 % CO_2. After 3 hours, lymphocytes in suspension were removed and adhered monocytes were gently detached and resuspended in PBS plus 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer).

Measurement of intracellular Ca^{2+} levels

MM6 cells or blood monocytes (3×10^7/ml PG buffer) were incubated with 2 µM Fura-2/AM for 30 min at 37°C. After washing, 3×10^6 cells/ml PG buffer were incubated in a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY) with continuous stirring. Two min prior stimulation, 1 mM $CaCl_2$ or 1 mM EDTA was added. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively, and $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985). F_{max} (maximal fluorescence) was obtained by lysing the cells with 1 % Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

SDS-PAGE and Western blotting

Prewarmed (37°C) MM6 cells were preincubated with the indicated concentrations of AKBA or vehicle (DMSO, final concentration ≤ 1 % (vol/vol)) for 5 min prior to stimulation with fMLP (1 µM) for 1 min at 37°C. The reaction was stopped by addition of the same volume of ice-cold 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (SDS-b), samples for SDS-PAGE (aliquots corresponding to 2×10^6 cells in 20 µl SDS-b) were prepared, and proteins were separated as described (Werz et al., 2002).

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Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. Western-blotting using phospho-specific antibodies (New England Biolabs (Beverly, MA), 1:1000 dilution, each) against pERK1/2 (Thr202/Tyr204) and pp38^{MAPK} (Thr180/Tyr182) was performed using a Li-Cor Odyssey^{TM} 2-color Western detection system (Li-Cor, Lincoln, NE), according to the instructions of the manufacturer. Alternatively, detection of immunoreactive proteins was performed as described previously using alkaline phosphatase-conjugated secondary AB (Werz et al., 2002).

Determination of IP_3 formation.

Prewarmed (37°C) MM6 cells (1.2×10^7/ml PGC buffer) were either preincubated with the indicated compounds (or vehicle, DMSO ≤ 1 % vol/vol) for 20 s and then subsequently stimulated with *m*-3M3FBS (100 µM) for 15 s, or directly stimulated with the indicated compounds for 15 s at 37°C. Incubations were stopped by the addition of 0.2 vol ice-cold $HClO_4$ (20 %, vol/vol) and kept on ice for 20 min. Further extraction and evaluation of IP_3 released into the medium was determined using an [^{3}H]-IP_3 Biotrak Assay System (Amersham, Little Chalfont, UK) according to the manufacturer's instructions. Data are expressed as percentage of vehicle-treated controls + S.E., n = 3 – 4. Statistical analysis was performed prior to normalisation, $p<0.05$ (*) or <0.01 (**).

Statistics

Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post-hoc tests. Where appropriate, Student´s *t* test for paired observations was applied. A *p* value of <0.05 (*) or <0.01 (**) was considered significant.

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Results

AKBA decreases basal $[Ca^{2+}]_i$ of resting MM6 cells.

In our previous reports, we showed that AKBA and KBA, but not AβBA and βBA (lacking the 11-keto group), cause a marked mobilization of Ca^{2+} in human isolated PMNL or in the granuloytic cell line HL-60 (Altmann et al., 2002; Altmann et al., 2004). Accordingly, 30 µM AKBA induced a rapid and pronounced elevation of $[Ca^{2+}]_i$ in PMNL, whereas AβBA was hardly effective (**fig. 1A**, left panel). In contrast, exposure of differentiated MM6 cells to AKBA (30 µM) resulted in a sudden drop of resting $[Ca^{2+}]_i$ from 155 +/- 8 to 73 +/- 7 nM (**fig. 1A**, right panel). This decrease in $[Ca^{2+}]_i$ was sustained, and markedly reduced levels of basal $[Ca^{2+}]_i$ were still detectable 20 minutes after exposure to AKBA (see below). In a previous study using MM6 cells (Feisst and Werz, 2004), we observed a similar drop of $[Ca^{2+}]_i$, when the PLC inhibitor U-73122 (3 µM) was added to the cells, which was confirmed in the present experiments (**fig. 1A**, right panel). Of interest, in PMNL, U-73122 (3 µM) caused no decrease in $[Ca^{2+}]_i$ (**fig. 1A**, left panel). The effect of AKBA on resting $[Ca^{2+}]_i$ was concentration-dependent and was clearly detectable already at 1 µM (**fig. 1B**). Combined addition of 30 µM AKBA plus 3 µM U-73122 gave no additive effects versus AKBA or U-73122 alone (not shown).

In analogy to PMNL, the effectiveness of the BAs to affect $[Ca^{2+}]_i$ in MM6 cells depended on the presence of the 11-keto group and the 3-*O*-acetyl moiety. Thus, the 11-keto-free counterpart of AKBA, namely AβBA (30 µM), hardly decreased $[Ca^{2+}]_i$, and KBA, lacking the 3-*O*-acetyl moiety, was less efficient than AKBA with respect to this response (**fig. 1A** and **C**). Finally, no effect was detectable for βBA (30 µM).

AKBA decreases elevated $[Ca^{2+}]_i$ in PAF-activated MM6 cells

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In order to evaluate if AKBA also affects elevated $[Ca^{2+}]_i$ induced by an agonist, MM6 cells were first treated with 100 nM PAF that raises $[Ca^{2+}]_i$, and AKBA, AβBA or U-73122 were added 50 sec later. Addition of AKBA or U-73122 evoked an immediate drop of $[Ca^{2+}]_i$ in a concentration-dependent manner (**fig. 2**). Notably, the minimum $[Ca^{2+}]_i$ attained after AKBA or U-73122 addition (78 +/- 10 and 99 +/- 9 nM, respectively) was lower than the basal Ca^{2+} levels prior stimulation with PAF (157 +/- 14 nM), and approached similar levels as found for cells exposed only to AKBA (73 +/- 7 nM) or U-73122 (88 +/- 8 nM), respectively (**fig. 2**, compare fig. 1C). Thus, AKBA decreases $[Ca^{2+}]_i$ in MM6 cells to a comparable extent as U-73122, apparently regardless of the activation state of the cell.

AKBA attenuates agonist-induced elevation of $[Ca^{2+}]_i$.

We sought to investigate if AKBA could also prevent agonist-induced elevations of $[Ca^{2+}]_i$. Agents that elevate $[Ca^{2+}]_i$ involving PLC/IP3 signalling (e.g. PAF, fMLP and *m*-3M3FBS) but also stimuli that raise $[Ca^{2+}]_i$ independent of the PLC/IP3 pathway like ionomycin or TG were added to MM6 cells that received BAs or U-73122, 20 s prior agonist addition. As shown in **fig. 3A**, AKBA and U-73122, but not AβBA, potently inhibited the subsequent Ca^{2+} mobilisation induced by the physiological agonists PAF or fMLP as well as by the direct PLC activator *m*-3M3FBS (Bae et al., 2003). The IC_{50} value for AKBA was in the range of 10 - 30 µM, depending on the stimulus. Representative $[Ca^{2+}]_i$ traces of PAF-stimulated samples are displayed in **fig. 3B**, left panel. In contrast, initial elevation of $[Ca^{2+}]_i$ induced by the ER/SR-Ca^{2+}-ATPase inhibitor thapsigargin (TG) or by the Ca^{2+}-ionophore ionomycin were not affected (**fig. 3A)**. Closer examination revealed that AKBA transforms the sustained elevation of $[Ca^{2+}]_i$ evoked by TG to a transient signal (**fig. 3B**, right panel).

The amplitudes of the Ca^{2+} transients of the physiological stimuli PAF and fMLP were still strongly attenuated by AKBA, when the preincubation period was expanded from 20 s to 20

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min (**fig. 3C**). In contrast, the suppressive effects of U-73122 were seemingly transient and markedly declined within 20 min for PAF-stimulated cells, but not so for cells challenged with fMLP (**fig. 3C**). Similarly, the decrease in basal $[Ca^{2+}]_i$ of (unstimulated) MM6 cells after prolonged (20 min) exposure to 30 µM AKBA was sustained, whereas for U-73122 the strong initial reduction of basal $[Ca^{2+}]_i$ detected right after exposure appeared to be transient and again was almost reversed after 20 min (**table 1**). Therefore, AKBA exerts a sustained suppression on Ca^{2+}-homeostasis by decreasing basal $[Ca^{2+}]_i$ as well as by attenuating (PLC/IP3-mediated) Ca^{2+} mobilisation.

It appeared possible that the suppressive effects of AKBA observed in MM6 could be related to the fact that MM6 is a human leukaemia cell line. Therefore, we used primary monocytes isolated from human blood to investigate effects of AKBA on $[Ca^{2+}]_i$. As shown in **fig. 3D**, AKBA (3 or 10 µM) rapidly decreased basal $[Ca^{2+}]_i$ and prevented PAF-induced Ca^{2+} mobilization in the same manner as observed for MM6 cells. AβBA was without effect and higher AKBA concentrations (≥ 30 µM) caused a rather slow but continuous increase in $[Ca^{2+}]_i$ seemingly related to cell lysis or unspecific toxic effects of the compound (not shown).

AKBA attenuates Ca^{2+} mobilisation from intracellular stores

Next, we investigated if AKBA may also affect the PAF-induced release of Ca^{2+} from intracellular stores, a process that is typically PLC/IP_3-dependent. MM6 cells were resuspended in Ca^{2+}-free buffer containing 1 mM EDTA and treated with AKBA (AβBA, or U-73122), followed by the addition of PAF after another 20 s. Neither AKBA (or AβBA) nor U-73122 exhibited an effect on basal $[Ca^{2+}]_i$ in resting cells under these conditions. However, AKBA or U-73122 reduced the release of Ca^{2+} from internal stores elicited by PAF (**fig. 4A, B**, left panel), although slightly higher concentrations of AKBA and U-73122 were required as compared to those needed to suppress total Ca^{2+} mobilisation in the presence of

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extracellular Ca^{2+}. Surprisingly, also Ca^{2+} mobilisation from internal storage sites induced by TG (**fig. 4B**, right panel) was partly antagonised by AKBA, implying that PLC inhibition may not be the sole mechanism by which AKBA affects $[Ca^{2+}]_i$, since TG-mediated Ca^{2+} mobilisation circumvents the PLC/IP_3 route.

AKBA attenuates fMLP-evoked MAPK activation

Cell activation, reflected by elevations in $[Ca^{2+}]_i$, is often accompanied by induction of signalling pathways leading to enhanced levels of phosphorylated members of the MAPK family (Belcheva and Coscia, 2002). The $p38^{MAPK}$ and ERKs are key effectors in the cellular signalling network of leukocytes. Addition of AKBA to resting MM6 cells caused a reduction of the basal levels of phosphorylated ERK-2, whereas phosphorylation of $p38^{MAPK}$ was not affected (**fig. 5A**). Note that phosphorylation state of MAPK solely indicates the activation of the kinases but might not exactly reflect the protein kinase activity towards its substrate(s).

Next, we examined whether AKBA prevents the activation of $p38^{MAPK}$ and ERKs induced by fMLP that, in contrast to PAF, causes prominent phosphorylation of the MAPK in MM6 cells (Poeckel, D. and Werz, O. (2005) unpublished results). Indeed, activation of ERKs was potently prevented by AKBA and again the amounts of phosphorylated ERK-2 were lower after preincubation with AKBA (10 and 30 µM) as compared to vehicle-treated control cells (**fig. 5B**). AKBA also attenuated fMLP-induced phosphorylation of $p38^{MAPK}$ (**fig. 5B**). It was shown that U-73122 substantially reduces the activation of $p38^{MAPK}$ in monocytic cells stimulated by lysophosphatidylcholine (Jing et al., 2000), and we found significant inhibition of fMLP-induced ERK2 activation by U-73122 in MM6 cells (not shown). AβBA, however, was inactive (not shown).

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Effects of boswellic acids on cellular PLC activity

In order to test if AKBA (in analogy to U-73122) inhibits cellular PLC activity, we assayed the effects of AKBA on the IP_3 formation in intact MM6 cells. Cellular PLC was directly activated using 100 µM *m*-3M3FBS, in order to obtain a prominent increase in IP_3 production (7.9-fold elevation, **fig. 6B**). In agreement with its ability to block total Ca^{2+} mobilisation, U-73122 (5 µM) inhibited *m*-3M3FBS-induced IP3 formation (~80 %, **fig. 6A)**. In contrast to its ability to decrease $[Ca^{2+}]_i$, AKBA failed to significantly suppress IP3 formation. Intriguingly, AβBA that hardly affected Ca^{2+} homeostasis, inhibited *m*-3M3FBS-induced IP3 generation (~50 %, **fig. 6A**).

We then examined if AKBA, AβBA or U-73122 could also affect the basal IP_3 turnover in resting cells. In contrast to *m*-3M3FBS-activated MM6 cells, no significant stimulatory effect was observed for U-73122 (5 µM) or AβBA. However, AKBA caused a concentration-dependent increase in IP_3 formation as compared to vehicle-treated control cells (**fig. 6B**). Possibly, this elevation of IP_3 might compromise the inhibitory effect of AKBA on the *m*-3M3FBS signal as shown in **fig 6A**, leading to an apparent weaker inhibition due to inherent AKBA-induced IP_3 production. Thus, a correlation between the suppression of Ca^{2+} homeostasis and inhibition of PLC by AKBA is not readily apparent, suggesting that AKBA operates at alternate targets than PLC to impair $[Ca^{2+}]_i$.

Inhibitors of plasma membrane Ca^{2+} channels mimic the effects of AKBA and abolish AKBA-induced decrease of $[Ca^{2+}]_i$.

The fact that AKBA attenuates the secondary phase of TG-induced Ca^{2+} mobilisation, which represents Ca^{2+} influx from the extracellular space, prompted us to elucidate if the AKBA-induced loss of intracellular (cytoplasmic) Ca^{2+} may be due to inhibition of plasma membrane Ca^{2+} influx channels such as store-operated Ca^{2+} channels (SOCC), non-selective cation

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channels (NSCC), or voltage-gated Ca^{2+} channels. SK&F96365, an inhibitor of NSCC and SOCC reduced basal $[Ca^{2+}]_i$ in MM6 cells, and was able to prevent the subsequent decrease of $[Ca^{2+}]_i$ induced by AKBA (**fig. 7**). In order to distinguish between NSCC and SOCC, we applied 2-APB (50 µM) that blocks SOCCs, and on the other hand LOE908 (10 µM) that selectively inhibits NSCC. Both, 2-APB as well as LOE908 decreased basal $[Ca^{2+}]_i$ and prevented the effects of AKBA (**fig. 7**). In contrast, inhibitors of voltage-gated Ca^{2+} channels (ω-conotoxin MVIIA (300 nM) or verapamil (1 µM) that block L-type or N-type channels, respectively), SR-Ca^{2+} release channels (neomycin, 10 µM) or blockers of the Na^{+}-Ca^{2+} exchanger (NCX; SEA0400 or KB-R7943, 10 µM, each) failed to significantly decrease basal $[Ca^{2+}]_i$ and to prevent effects of AKBA (not shown). Together, these results indicate that AKBA may mediate the decrease of $[Ca^{2+}]_i$ by blocking Ca^{2+} influx from the extracellular space via inhibition of SOCC/NSCC.

Discussion

AKBA and KBA were shown to induce Ca^{2+} mobilisation and activation of MAPK in primary PMNL and granulocytic HL-60 cells, involving pertussis toxin-sensitive proximal signalling pathways (Altmann et al., 2002; Altmann et al., 2004). Activation of these central signalling events were linked to typical functional responses of granulocytes including peroxide formation and enhanced metabolism of AA, in particular an increased activity of 5-LO was evident (Altmann et al., 2004). Such an activation of granulocytes opposes the general observation that extracts of *B. serrata* or isolated BAs exert anti-inflammatory properties in several cellular experimental settings (Krieglstein et al., 2001; Syrovets et al., 2005) or animal models (Sharma et al., 1989; Gupta et al., 1994), and finally also in studies on human subjects (Gupta et al., 1998; Gerhardt et al., 2001).

Recently, Syrovets et al. showed that in activated human monocytes, BAs down regulate TNFα expression via a direct inhibition of I-κB kinases, providing a molecular basis for the anti-inflammatory properties of BAs (Syrovets et al., 2005). The result from the present investigation focusing on central signalling pathways in monocytes provides additional evidence for an anti-inflammatory implication of AKBA at the cellular level. Thus, AKBA decreased the basal $[Ca^{2+}]_i$, prevented agonist-induced Ca^{2+} mobilisation, and blocked the activation of ERK1/2 and $p38^{MAPK}$, signalling events that are determinants for typical functional monocyte/macrophage responses (Gijon and Leslie, 1999; Chen et al., 2001). Interestingly, TNFα generation and NF-κB activation in monocytic cells may depend on Ca^{2+} (Pollaud-Cherion et al., 1998; See et al., 2004), providing a possible link between interference with Ca^{2+} and downregulation of NF-κB and TNFα.

Apparently, in view of the opposite, agonistic effects on PMNL and HL-60 cells, AKBA and KBA exert disparate effects on certain cellular processes, depending on the cell type. Thus, in monocytic cells, AKBA may be regarded as pharmacologically active compound that

suppresses important signalling events implying anti-inflammatory functionality. PMNL that are terminally differentiated are involved in acute inflammatory responses, whereas monocytes act more in chronic inflammation and can undergo differentiation prior to function. Indeed, opposite effects on Ca^{2+} homeostasis in analogy to AKBA are obvious in leukocytes exposed to arachidonic acid that decreases $[Ca^{2+}]_i$ in peritoneal macrophages (Randriamampita and Trautmann, 1990) and in MM6 cells (Poeckel, D. and Werz, O. (2005) unpublished results) but on the other hand evokes Ca^{2+} mobilization in PMNL (Naccache et al., 1989).

Many effector enzymes like phospholipases, 5-LO and protein kinases respond to and are regulated by an elevation of $[Ca^{2+}]_i$, leading to functional monocyte responses including lipid mediator and superoxide release, degranulation, and cytokine generation (Bernardo et al., 1988; Pollaud-Cherion et al., 1998). Among the four major β-configurated BAs present in ethanolic extracts of *B. serrata* gum, AKBA was most potent, whereas the 11-methylene derivatives were hardly active and also the absence of the 3-*O*-acetyl group led to a loss of efficacy. Similarly, for interference with so far all defined molecular pharmacological targets, i.e. 5-LO (Safayhi et al., 1992), human leukocyte elastase (Safayhi et al., 1997), toposiomerases I and II (Syrovets et al., 2000)], as well as I-κB kinases (Syrovets et al., 2005), AKBA possesses the highest potency, being of considerable pharmacological interest (Ammon, 2002).

The effects of AKBA in MM6 cells showed similar characteristics as the PLC inhibitor U-73122 (Bleasdale et al., 1990) that was found to block acute and chronic inflammatory responses *in vivo* (Hou et al., 2004). Indeed, both U-73122 and AKBA rapidly decreased the basal $[Ca^{2+}]_i$ of resting cells but also caused an immediate drop of the elevated $[Ca^{2+}]_i$ after challenge with PAF, displaying comparable kinetics. Moreover, both agents reduced agonist-evoked Ca^{2+} mobilisation which in fact is a characteristics for monocyte activation by external

stimuli (Kim et al., 1992; Bernardo et al., 1997; Li et al., 2002). Such Ca^{2+}-antagonising activity of AKBA or U-73122 was evident for agonists (fMLP, PAF, or *m*-3M3FBS) that act via the PLC/IP3 pathway. In contrast, initial Ca^{2+} fluxes induced by the ER/SR-Ca^{2+}-ATPase inhibitor TG or by the Ca^{2+}-ionophore ionomycin, that both circumvent PLC/IP3 for Ca^{2+} mobilisation (Gouy et al., 1990), were unaffected by either U-73122 or AKBA. Experiments conducted to determine the duration of the Ca^{2+} suppressing effects, either in resting or in agonist-challenged cells, revealed rather transient efficacy of U-73122, whereas AKBA-mediated antagonism was sustained and long-lasting, implying that the compounds most likely operate through differing mechanisms.

Another common feature of U-73122 and AKBA was their ability to inhibit the PAF-induced release of Ca^{2+} from internal storage sites. Hence, based on the inhibitory profile and characteristics to affect Ca^{2+} homeostasis, it first appeared reasonable that the Ca^{2+}-modulating effects of AKBA could be due to interference with PLC, which is a defined molecular target of U-73122 (Bleasdale et al., 1990). On the other hand, interference of AKBA with the IP3 receptor could be a plausible explanation. Surprisingly, however, AKBA significantly inhibited TG-induced Ca^{2+} mobilisation from internal stores, and also the sustained elevation of $[Ca^{2+}]_i$ of TG-treated cells in Ca^{2+}-containing buffer, suggesting that AKBA may influence Ca^{2+} homeostasis, at least in part, independent of PLC or IP3. An important finding that favours a PLC-independent mechanism is the failure of AKBA to efficiently suppress the release of intracellular IP3. Strikingly, in contrast to AKBA, AβBA clearly failed to counteract *m*-3M3FBS-induced Ca^{2+} mobilisation, even though it was more efficient than AKBA in inhibiting *m*-3M3FBS-evoked IP_3 formation. Based on these discrepancies, inhibition of PLC is no satisfying explanation for the potent impairment of $[Ca^{2+}]_i$ induced by AKBA. This hypothesis is further supported by the fact that AKBA on one hand even slightly increased basal IP3 levels about 2-fold which should actually lead to Ca^{2+}

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release from internal storage sites. However, in contrast, there is a strong decrease in the basal $[Ca^{2+}]_i$ under these conditions.

The AKBA-induced loss of intracellular (cytoplasmic) Ca^{2+} may result from different processes such as extrusion of intracellular Ca^{2+} to the extracellular space, stimulation of Ca^{2+} storage (uptake) into intracellular sites (e.g. by activation of a Ca^{2+}/ATPase), or interference with ion channels allowing Ca^{2+}-influx. Our studies using selective inhibitors of various plasma membrane Ca^{2+} influx channels imply that AKBA might act (at least in part) by inhibition of SOCC and/or NSCC. Thus, inhibitors of SOCC and/or NSCC mimicked the loss of $[Ca^{2+}]_i$ observed with AKBA and were able to inhibit the subsequent decrease of $[Ca^{2+}]_i$ induced by AKBA. Of interest, also for U-73122 inhibition of plasma membrane Ca^{2+} channels has been accounted for reduced $[Ca^{2+}]_i$ (see Feisst et al., 2005 and references therein). In contrast, voltage-gated N- and L-type Ca^{2+} channels, SR-Ca^{2+} release channels (neomycin) or the Na^+-Ca^{2+} exchanger do not seem to mediate the effects of AKBA. It is conceivable that a block of SOCC/NSCC may shift the balance between Ca^{2+} influx and Ca^{2+} extrusion towards predominant extrusion that, as a result, leads to impaired $[Ca^{2+}]_i$. However, more detailed experiments are required in order to elucidate the molecular targets and mechanisms underlying the complex regulation of Ca^{2+} homeostasis by AKBA in MM6 cells, which would go beyond the scope of this study.

Besides antagonising Ca^{2+}, AKBA potently prevented fMLP-induced activation of $p38^{MAPK}$ and ERKs. These MAPK pathways play pivotal roles in the transduction of external mediators to many cellular processes and are strongly implicated in inflammatory disorders (Herlaar and Brown, 1999; Johnson and Druey, 2002). Among the pharmacological strategies for intervention with inflammation, inhibitors of ERKs may possess potential for the treatment of inflammatory and neuropathic pain (Ji, 2004). $p38^{MAPK}$ inhibitors have been developed to treat for example rheumatoid arthritis (Pargellis and Regan, 2003) and Crohn´s disease

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(Hommes et al., 2002), inflammatory disorders which in fact have been successfully treated with *B. serrata* extracts (Gerhardt et al., 2001). Of interest, recently also U-73122 was shown to reduce lysophosphatidylcholine-induced $p38^{MAPK}$ activation in monocytic THP-1 cells (Jing et al., 2000).

In summary, our data show that AKBA is capable to suppress central signalling events in human monocytic cells, typically important for functional monocyte responses at inflammatory sites. These findings may be added to the list of pharmacological actions of BAs assumed to contribute to the effects of *B. serrata* extracts observed in animal models and in clinical studies of humans, and may be another step forward to the elucidation of the cellular and molecular basis of the anti-inflammatory properties of BAs.

References

Altmann A, Fischer L, Schubert-Zsilavecz M, Steinhilber D and Werz O (2002) Boswellic acids activate p42(MAPK) and p38 MAPK and stimulate Ca(2+) mobilization. *Biochem Biophys Res Commun* **290**:185-190.

Altmann A, Poeckel D, Fischer L, Schubert-Zsilavecz M, Steinhilber D and Werz O (2004) Coupling of boswellic acid-induced Ca2+ mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes. *Br J Pharmacol* **141**:223-232.

Ammon HP (2002) [Boswellic acids (components of frankincense) as the active principle in treatment of chronic inflammatory diseases]. *Wien Med Wochenschr* **152**:373-378.

Bae YS, Lee TG, Park JC, Hur JH, Kim Y, Heo K, Kwak JY, Suh PG and Ryu SH (2003) Identification of a compound that directly stimulates phospholipase C activity. *Mol Pharmacol* **63**:1043-1050.

Belcheva MM and Coscia CJ (2002) Diversity of G protein-coupled receptor signaling pathways to ERK/MAP kinase. *Neurosignals* **11**:34-44.

Bernardo J, Billingslea AM, Ortiz MF, Seetoo KF, Macauley J and Simons ER (1997) Adherence-dependent calcium signaling in monocytes: induction of a CD14-high phenotype, stimulus-responsive subpopulation. *J Immunol Methods* **209**:165-175.

Bernardo J, Brink HF and Simons ER (1988) Time dependence of transmembrane potential changes and intracellular calcium flux in stimulated human monocytes. *J Cell Physiol* **134**:131-136.

Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ and Bunting S (1990) Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharmacol Exp Ther* **255**:756-768.

Chen BC, Hsieh SL and Lin WW (2001) Involvement of protein kinases in the potentiation of lipopolysaccharide-induced inflammatory mediator formation by thapsigargin in peritoneal macrophages. *J Leukoc Biol* **69**:280-288.

Feisst C, Albert D, Steinhilber D and Werz O (2005) The Aminosteroid Phospholipase C Antagonist U-73122 (1-[6-[[17-{beta}-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyr role-2,5-dione) Potently Inhibits Human 5-Lipoxygenase in Vivo and in Vitro. *Mol Pharmacol* **67**:1751-1757.

Feisst C and Werz O (2004) Suppression of receptor-mediated Ca2+ mobilization and functional leukocyte responses by hyperforin. *Biochem Pharmacol* **67**:1531-1539.

Gerhardt H, Seifert F, Buvari P, Vogelsang H and Repges R (2001) [Therapy of active Crohn disease with Boswellia serrata extract H 15]. *Z Gastroenterol* **39**:11-17.

Gijon MA and Leslie CC (1999) Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J Leukoc Biol* **65**:330-336.

Gouy H, Cefai D, Christensen SB, Debre P and Bismuth G (1990) Ca2+ influx in human T lymphocytes is induced independently of inositol phosphate production by mobilization of intracellular Ca2+ stores. A study with the Ca2+ endoplasmic reticulum-ATPase inhibitor thapsigargin. *Eur J Immunol* **20**:2269-2275.

Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**:3440-3450.

Gupta I, Gupta V, Parihar A, Gupta S, Ludtke R, Safayhi H and Ammon HP (1998) Effects of Boswellia serrata gum resin in patients with bronchial asthma: results of a double-blind, placebo-controlled, 6-week clinical study. *Eur J Med Res* **3**:511-514.

Gupta OP, Sharma N and Chand D (1994) Application of papaya latex-induced rat paw inflammation: model for evaluation of slowly acting antiarthritic drugs. *J Pharmacol Toxicol Methods* **31**:95-98.

Herlaar E and Brown Z (1999) p38 MAPK signalling cascades in inflammatory disease. *Mol Med Today* **5**:439-447.

Hommes D, van den Blink B, Plasse T, Bartelsman J, Xu C, Macpherson B, Tytgat G, Peppelenbosch M and Van Deventer S (2002) Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. *Gastroenterology* **122**:7-14.

Hou C, Kirchner T, Singer M, Matheis M, Argentieri D and Cavender D (2004) In vivo activity of a phospholipase C inhibitor, 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole -2,5-dione (U73122), in acute and chronic inflammatory reactions. *J Pharmacol Exp Ther* **309**:697-704.

Jauch J and Bergmann J (2003) An efficient method for the large-scale preparation of 3-O-acetyl-11-oxo-beta-boswellic acid and other boswellic acids. *Eur J Org Chem* 4752-4756.

Ji RR (2004) Mitogen-activated protein kinases as potential targets for pain killers. *Curr Opin Investig Drugs* **5**:71-75.

Jing Q, Xin SM, Zhang WB, Wang P, Qin YW and Pei G (2000) Lysophosphatidylcholine activates p38 and p42/44 mitogen-activated protein kinases in monocytic THP-1 cells, but only p38 activation is involved in its stimulated chemotaxis. *Circ Res* **87**:52-59.

Johnson EN and Druey KM (2002) Heterotrimeric G protein signaling: role in asthma and allergic inflammation. *J Allergy Clin Immunol* **109**:592-602.

Kim E, Enelow RI, Sullivan GW and Mandell GL (1992) Regional and generalized changes in cytosolic free calcium in monocytes during phagocytosis. *Infect Immun* **60**:1244-1248.

Krieglstein CF, Anthoni C, Rijcken EJ, Laukotter M, Spiegel HU, Boden SE, Schweizer S, Safayhi H, Senninger N and Schurmann G (2001) Acetyl-11-keto-beta-boswellic acid, a constituent of a herbal medicine from Boswellia serrata resin, attenuates experimental ileitis. *Int J Colorectal Dis* **16**:88-95.

Li SW, Westwick J and Poll CT (2002) Receptor-operated Ca2+ influx channels in leukocytes: a therapeutic target? *Trends Pharmacol Sci* **23**:63-70.

Naccache PH, McColl SR, Caon AC and Borgeat P (1989) Arachidonic acid-induced mobilization of calcium in human neutrophils: evidence for a multicomponent mechanism of action. *Br J Pharmacol* **97**:461-468.

Pargellis C and Regan J (2003) Inhibitors of p38 mitogen-activated protein kinase for the treatment of rheumatoid arthritis. *Curr Opin Investig Drugs* **4**:566-571.

Pollaud-Cherion C, Vandaele J, Quartulli F, Seguelas MH, Decerprit J and Pipy B (1998) Involvement of calcium and arachidonate metabolism in acetylated-low-density-lipoprotein-stimulated tumor-necrosis-factor-alpha production by rat peritoneal macrophages. *Eur J Biochem* **253**:345-353.

Randriamampita C and Trautmann A (1989) Biphasic increase in intracellular calcium induced by platelet-activating factor in macrophages. *FEBS Lett* **249**:199-206.

Randriamampita C and Trautmann A (1990) Arachidonic acid activates Ca2+ extrusion in macrophages. *J Biol Chem* **265**:18059-18062.

Safayhi H, Mack T, Sabieraj J, Anazodo MI, Subramanian LR and Ammon HP (1992) Boswellic acids: novel, specific, nonredox inhibitors of 5-lipoxygenase. *J Pharmacol Exp Ther* **261**:1143-1146.

Safayhi H, Rall B, Sailer ER and Ammon HP (1997) Inhibition by boswellic acids of human leukocyte elastase. *J Pharmacol Exp Ther* **281**:460-463.

Safayhi H and Sailer ER (1997) Anti-inflammatory actions of pentacyclic triterpenes. *Planta Med* **63**:487-493.

See V, Rajala NK, Spiller DG and White MR (2004) Calcium-dependent regulation of the cell cycle via a novel MAPK--NF-kappaB pathway in Swiss 3T3 cells. *J Cell Biol* **166**:661-672.

Sharma ML, Bani S and Singh GB (1989) Anti-arthritic activity of boswellic acids in bovine serum albumin (BSA)-induced arthritis. *Int J Immunopharmacol* **11**:647-652.

Syrovets T, Buchele B, Gedig E, Slupsky JR and Simmet T (2000) Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and IIalpha. *Mol Pharmacol* **58**:71-81.

Syrovets T, Buchele B, Krauss C, Laumonnier Y and Simmet T (2005) Acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF-alpha induction in monocytes by direct interaction with IkappaB kinases. *J Immunol* **174**:498-506.

Werz O, Burkert E, Samuelsson B, Rådmark O and Steinhilber D (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* **99**:1044-1052.

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Figure Legends

Fig. 1 Effects of BAs and U-73122 on basal $[Ca^{2+}]_i$ in resting PMNL and MM6 cells. Fura-2 loaded PMNL (10^7/ml PG buffer, **A,** left panel) or MM6 cells (3×10^6/ml PG buffer, **A**, right panel, **B, C**), were supplemented with 1 mM $CaCl_2$, 2 min prior stimulation, and $[Ca^{2+}]_i$ was determined. (**A**) The addition of AKBA, AβBA (30 µM, each), U-73122 (3 µM), or vehicle (DMSO) to resting PMNL (left panel) or MM6 cells (right panel) is indicated by the arrow. Traces are representative for 3 – 6 independent recordings. (**B**) Concentration-response curves of AKBA on $[Ca^{2+}]_i$ in resting MM6 cells. The minimum $[Ca^{2+}]_i$ within 30 s after addition was determined. (**C**) Structure-activity relationship of BAs on $[Ca^{2+}]_i$ of resting MM6 cells. Vehicle (veh, DMSO), AKBA, KBA, AβBA, βBA (30 µM, each), or U-73122 (3 µM) were added as described above. The amplitude or an average value of the Ca^{2+} decrease (in nM) was determined. Values in (B, C) are given as mean + S.E., n = 4 - 5. Statistically different values compared to vehicle-treated controls are marked (**, $p<0.01$).

Fig. 2 Effects of BAs and U-73122 on elevated $[Ca^{2+}]_i$ in stimulated MM6 cells. MM6 cells were prepared as described in fig. 1. PAF (0.1 µM) was added as indicated, followed by vehicle (veh) or AKBA (3 - 30 µM, left panel); AβBA (30 µM) or U-73122 (1 - 10 µM, right panel) after another 50 s. Curves are representative for 3-5 independent determinations.

Fig. 3 AKBA and U-73122 antagonize agonist-induced Ca^{2+} mobilisation. MM6 cells were prepared as described in fig. 1. (**A**) Cells were treated with vehicle (v), AKBA (3, 10, 30 µM), AβBA (Aβ, 30 µM), or U-73122 (U, 3 µM) followed by the addition of PAF (0.1 µM), fMLP (0.1 µM), *m*-3M3FBS (50 µM), thapsigargin (TG, 0.1 µM), or ionomycin (0.2 µM) after 20 s as indicated. The amplitude of the agonist-induced elevation of $[Ca^{2+}]_i$ was

determined. Values are given as mean + S.E., n = 4 - 5, and compared to the positive controls, $p<0.05$ (*) or <0.01 (**). (**B**) Original Ca^{2+} recordings of measurements conducted for fig 3A. Left panel: cells were pretreated with vehicle (veh), AKBA (3, 10, 30 µM), AβBA (30 µM), or U-73122 (3 µM) for 20 s and PAF (0.1 µM) was added as indicated by the arrows. Right panel: cells were pretreated with vehicle (veh), AKBA (30 µM), AβBA (30 µM), or U-73122 (3 µM) for 20 s and thapsigargin (TG, 0.1 µM) was added as indicated by the arrows. Curves are representative for 3 - 5 independent determinations. (**C**) Efficacy of AKBA and U-73122 to inhibit agonist-induced Ca^{2+} mobilisation depends on the preincubation period. Cells were incubated with AKBA (30 µM) or U-73122 (3 µM). Then, PAF or fMLP (0.1 µM, each) were added as indicated either 20 s (black bars) or 20 min (white bars) after AKBA or U-73122. The resulting maximum increase in $[Ca^{2+}]_i$ was determined and compared to vehicle-treated controls, given as percentage of control +/- S.E., n = 3-5. Statistical analysis was performed prior to normalisation, $p<0.05$ (*) or $p<0.01$ (**). (**D**) Fura-2 loaded primary monocytes (3×10^6/ml PGC buffer), freshly isolated from human blood, were treated with vehicle (v) or AKBA (3 or 10 µM), followed by the addition of PAF (0.1 µM) after 20 s, as indicated by the arrows. Curves are representative for 3 independent determinations.

Fig. 4 Effects of BAs on Ca^{2+} release from internal stores. MM6 cells were prepared as described in fig. 1, except that 1 mM EDTA was added instead of 1 mM Ca^{2+}. (**A**) Original Ca^{2+} recordings of samples stimulated by PAF (0.1 µM) after preincubation with vehicle (veh), AKBA (3, 10, 30 µM), AβBA (30 µM), or U-73122 (3 µM) for 20 s. Curves are representative for 3 - 4 independent determinations. (**B**) Cells were treated with vehicle (v), AKBA (3, 10, 30 µM), AβBA (Aβ, 30 µM), or U-73122 (U, 3 µM) followed by the addition of PAF (0.1 µM, left panel), or thapsigargin (TG, 0.1 µM, right panel). The amplitude of the

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stimulus-induced elevation of $[Ca^{2+}]_i$ was determined. Values are given as mean + S.E., n = 4, $p<0.05$ (*) or <0.01 (**).

Fig. 5 AKBA attenuates the activation of ERK1/2 and p38^{MAPK}. (**A**) Prewarmed MM6 cells (2×10^6/100 µl PGC buffer) were incubated with the indicated concentrations of AKBA or vehicle (DMSO) for 5 min. (**B**) Prewarmed MM6 cells were first incubated with the indicated concentrations of AKBA or vehicle (DMSO) for 15 min prior to stimulation with fMLP (1 µM) at 37°C for 1 min. Reactions were terminated by addition of equal volumes of SDS-b. Samples were subjected to SDS-PAGE and Western blotting using phospho-specific antibodies against the dually phosphorylated form of the MAPKs. Samples were concurrently analysed for p38^{MAPK} and ERK1/2 activation using the 2-color Western detection system of Li-Cor (Odyssey^{TM}), hence loading control (L.C.) bands refer to both antibodies. The results shown are representative of at least 3 independent experiments.

Fig. 6 Effects of BAs on IP_3 formation. (**A**) Prewarmed MM6 cells (1.2×10^7/ml PGC buffer) were treated with vehicle (DMSO), AKBA (30 µM), AβBA (30 µM), or U-73122 (5 µM) for 20 s prior to stimulation with *m*-3M3FBS (100 µM) for 15 s. Incubations were stopped by the addition of 0.2 vol ice-cold $HClO_4$ (20 %, v/v) and extraction and evaluation of IP_3 released was determined according to the manufacturer's instructions (IP_3 [^{3}H] Biotrak Assay System, Amersham, Little Chalfont, UK). Data are expressed as percentage of vehicle-treated (*m*-3M3FBS-stimulated) control + S.E., n = 3 – 4. Statistical analysis was performed prior to normalisation, $p<0.05$ (*) or <0.01 (**). (**B**) Prewarmed MM6 cells (1.2×10^7/ml PGC buffer) were incubated with vehicle (DMSO), AKBA (3, 10, 30 µM), AβBA (30 µM), U-73122 (5 µM), or *m*-3M3FBS (100 µM) for 15 s. Incubations were stopped by the addition of 0.2 vol ice-cold $HClO_4$ (20 %, v/v). The subsequent extraction procedure is identical to the

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description in (A). Data are expressed as percentage of vehicle-treated (unstimulated) control + S.E., n = 3 – 4. Statistical analysis was performed prior to normalisation, $p<0.05$ (*) or <0.01 (**).

Fig. 7 Effects of plasma membrane Ca^{2+} channel inhibitors on Ca^{2+} homeostasis and on the actions of AKBA. Fura-2 loaded MM6 cells (3×10^6/ml PG buffer) were preincubated with vehicle (veh, DMSO), SK&F96365 (10 or 30 µM), 2-APB (50 µM), or LOE908 (10 µM) for 2 min at 37°C in the presence of 1 mM $CaCl_2$. Then, cells were stimulated with AKBA (30 µM) and $[Ca^{2+}]_i$ was determined. Traces are representative for 3 - 4 independent determinations.

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Table 1: AKBA but not U-73122 causes sustained depression of $[Ca^{2+}]_i$.

MM6 cells in PGC buffer were preincubated with vehicle (DMSO), AKBA, AβBA, or U-73122 for 20 s or 20 min, and $[Ca^{2+}]_i$ was determined, given as mean +/- S.E.. n = 4-6, p<.01 (**).

agonist	$[Ca^{2+}]_i$ (nM) after addition of agonist	
	20 s	20 min
vehicle (DMSO)	162 ± 4	157 ± 14
AKBA (30 µM)	73 ± 8 (**)	83 ± 5 (**)
AβBA (30 µM)	139 ± 4	146 ± 16
U-73122 (3 µM)	87 ± 10 (**)	132 ± 13

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Figure 1

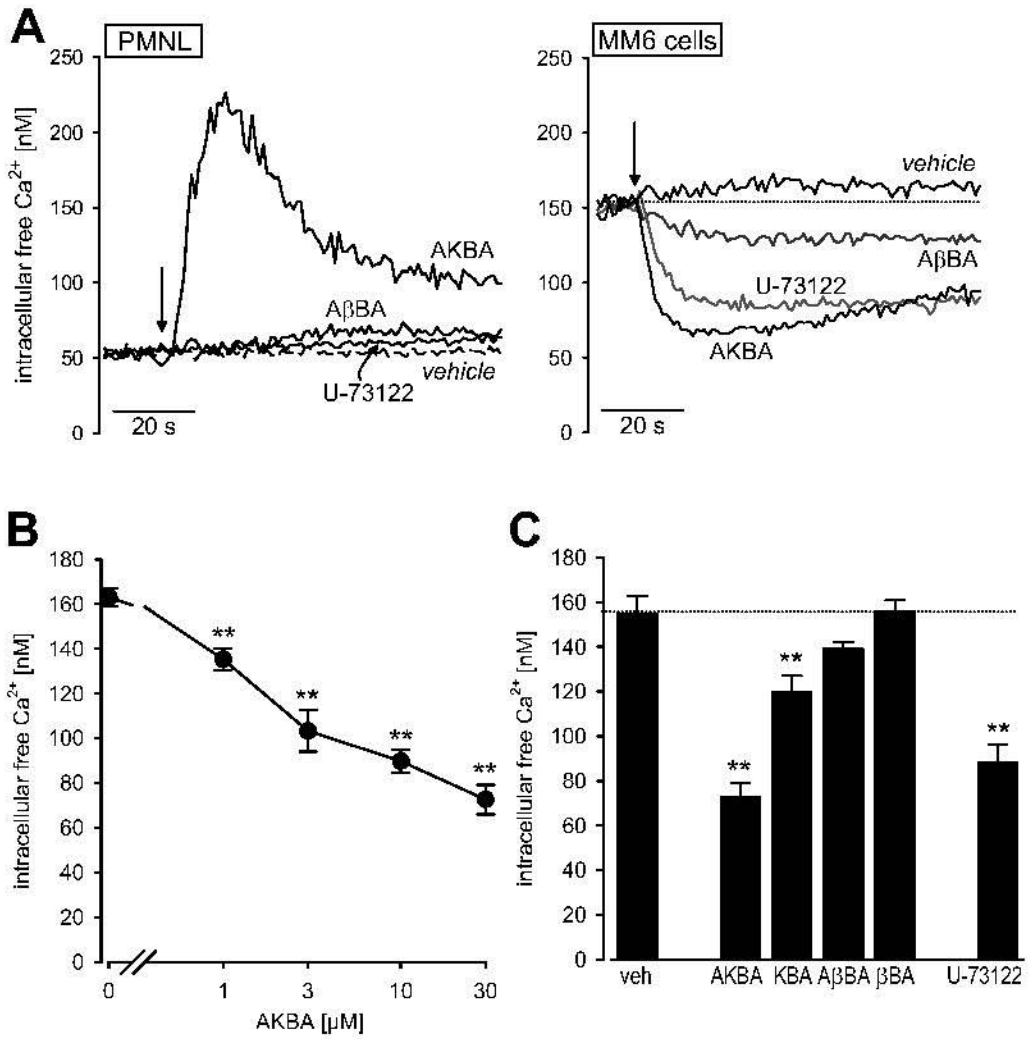

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Figure 2

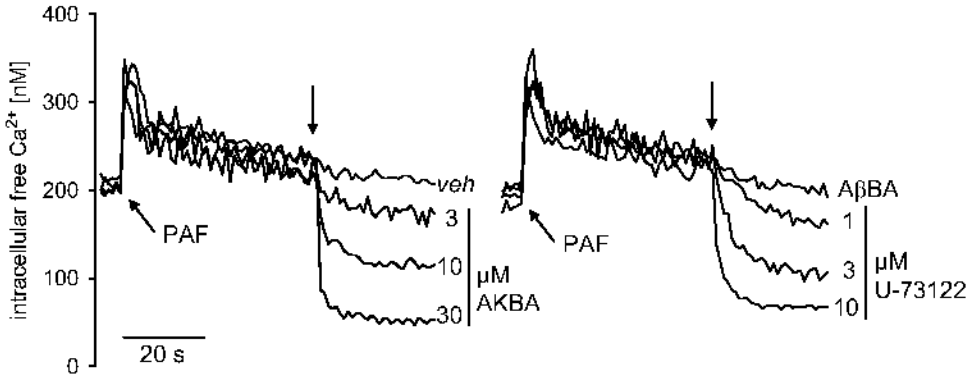

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Figure 3

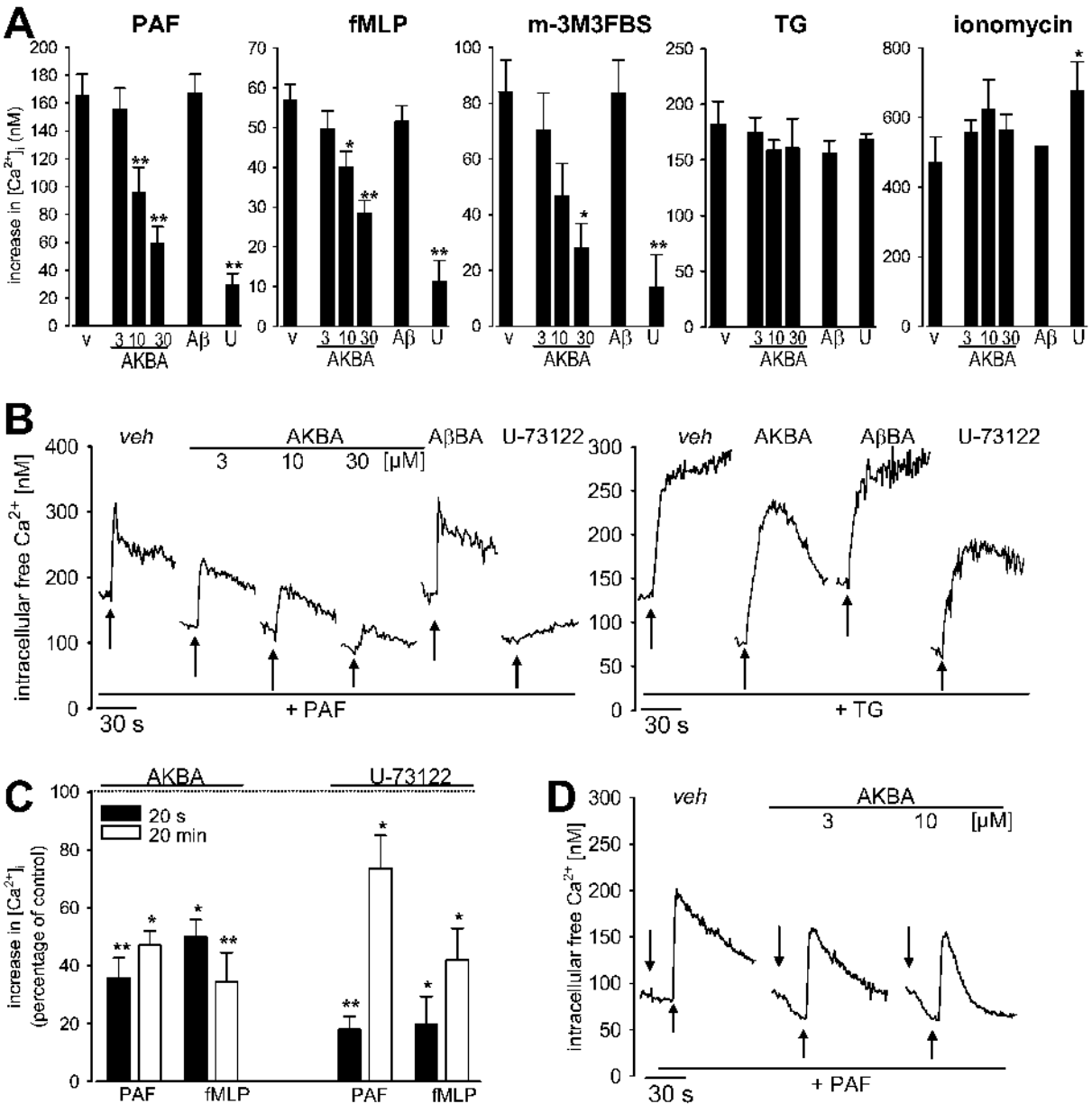

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Figure 4

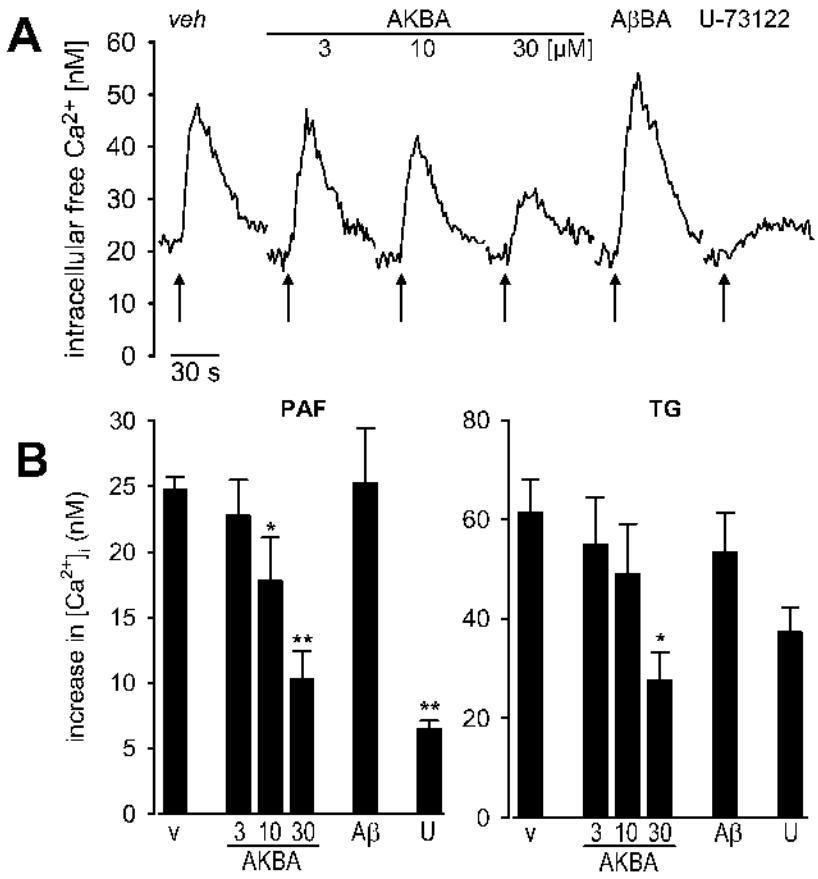

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Figure 5

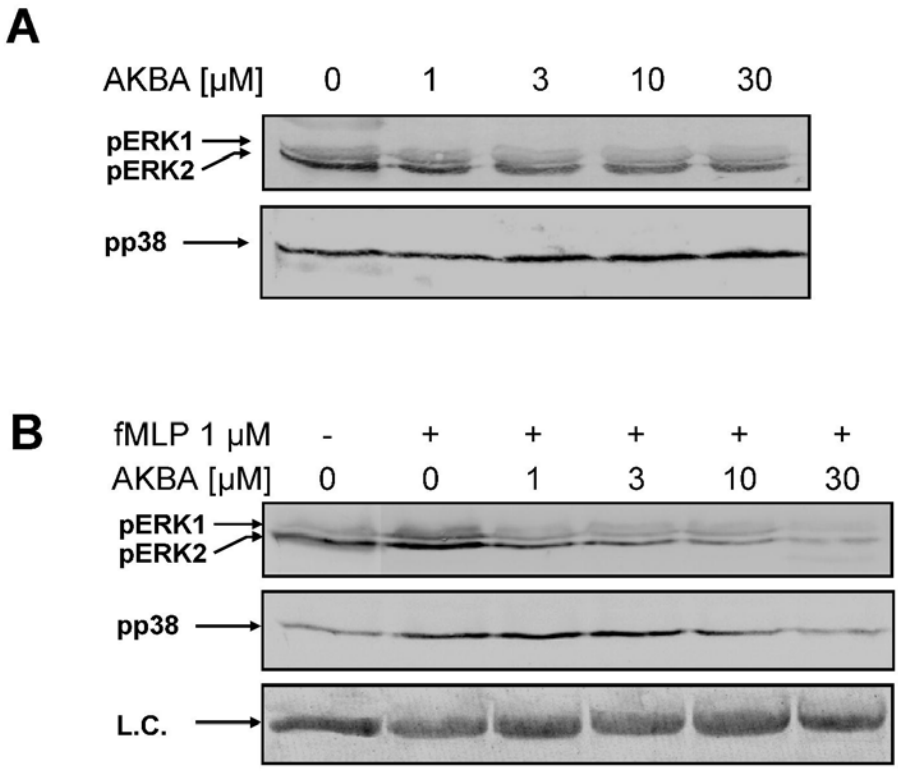

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Figure 6

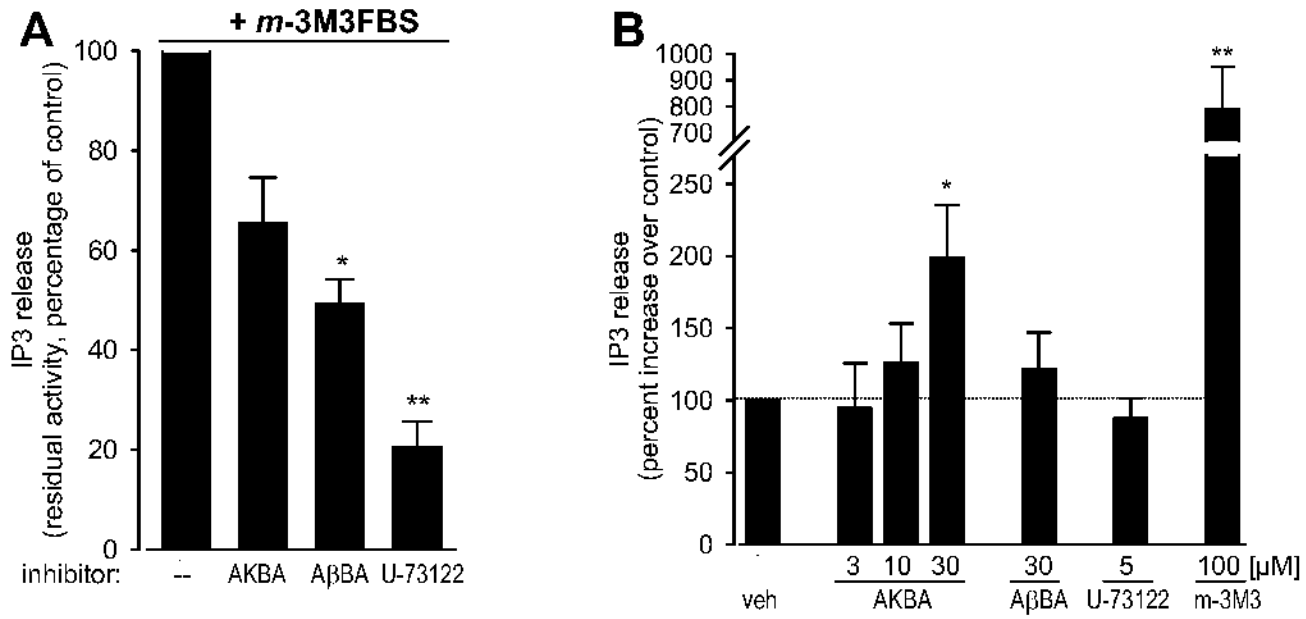

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Figure 7