3-O-Acetyl-11-keto-boswellic Acid Decreases Basal Intracellular Ca\(^{2+}\) Levels and Inhibits Agonist-Induced Ca\(^{2+}\) Mobilization and Mitogen-Activated Protein Kinase Activation in Human Monocytic Cells

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ABSTRACT

Previously, we showed that 11-keto-boswellic acid and 3-O-acetyl-11-keto-BA (AKBA) stimulate Ca\(^{2+}\) mobilization and activate mitogen-activated protein kinases (MAPKs) in human polymorphonuclear leukocytes (PMNLs). Here, we addressed the effects of boswellic acids on the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and on the activation of p38MAPK and extracellular signal-regulated kinase (ERK) in the human monocytic cell line Mono Mac (MM) 6. In contrast to PMNLs, AKBA concentration dependently (1–30 \(\mu\)M) 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\(^{2+}\)]\(_i\) by AKBA, which was induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP), PAF, or by the direct phospholipase C activator 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide, 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 p38MAPK and ERKs in FMLP-stimulated MM6 cells. Although the effects of AKBA could be mimicked by the putative phospholipase C (PLC) inhibitor U-73122 (1-[6-[[17β-methoxyestr-1,3,5(10)-trien-17-yl][amino]hexyl]-1H-pyrole-2,5-dione), AKBA appears to operate independent of PLC activity since the release of intracellular inositol-1,4,5-trisphosphate evoked by 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide 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 signaling 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.

Boswellia serrata 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. serrata 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 to elucidate the cellular and molecular mecha-
nisms underlying the clinical effects of BAs identified 5-lipoxygenase (5-LO) (Safayhi et al., 1992), human leukocyte elastase (Safayhi et al., 1997), topoisomerase I and II (Syroyvets et al., 2000), and IkB kinases (Syroyvets 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 TNFα expression in activated monocytes (Syroyvets 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 signaling systems that operate at multiple levels. Elevation of the intracellular Ca²⁺ concentration ([Ca²⁺]i) is one central signaling 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-methylionyl-leucyl-phenylalanine (fMLP), increase the [Ca²⁺]i in monocytes/macrophages, which is composed of a rapid release of Ca²⁺ from intracellular stores and a Ca²⁺ influx through plasma membrane Ca²⁺ channels (Randriamampita and Trautmann, 1989). Besides Ca²⁺, protein phosphorylation is a common signal transduction mechanism integrating extracellular inflammatory signals into leukocyte functions; 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 to intervene with inflammatory disorders.

We have recently shown that 11-keto-BAs can activate MAPK and induce Ca²⁺ mobilization in human isolated polymorphonuclear leukocytes (PMNLs) and granulocytic HL-60 cells, which could be linked to various functional responses, including release of arachidonic acid, increased formation of leukotrienes, and generation of reactive oxygen species (Altman et al., 2002, 2004). Since monocytes play key roles in the course of inflammatory processes, we examined the effect of BAs on the Ca²⁺ homeostasis and MAPK pathways in human monocyte Mono Mac (MM) 6 cells. Interestingly, in contrast to PMNLs or HL-60 cells, AKBA exerted opposite effects in MM6 cells, inasmuch as it decreased basal [Ca²⁺]i, inhibited agonist-induced Ca²⁺ mobilization, and blocked agonist-induced activation of p38MAPK and ERKs. These findings support an anti-inflammatory potential of AKBA.

**Materials and Methods**

**Materials.** BAs were prepared as described previously (Jauch and Bergmann, 2003). 2,4,6-Trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonylamide (m-3M3FBS) was a generous gift from Dr. T.G. Lee (SIGMOL, Pohang, Korea). U-73122 and SK&F96365 were purchased from Calbiochem (San Diego, CA); Fura-2/AM was from Alexis Corporation (Läufelfingen, Switzerland), PAP was from Cayman Chemical (Ann Arbor, MI), 2-aminoethoxydiphenylborate (2-APB) was from Tocris Cookson Inc. (Bristol, UK), and all other chemicals were obtained from Sigma Chemie (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, 1X nonessential amino acids, 1 mM oxalacetic acid, and 10 μg/ml bovine insulin. All cultures were seeded at a density of 2 × 10⁶ 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 (200g, 10 min at room temperature) and washed once in phosphate-buffered saline (PBS), pH 7.4. To exclude toxic effects of BAs during various incubation periods, the viability of MM6 cells was analyzed 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 PMNLs were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany) as described (Wertz et al., 2002). In brief, venous blood was taken from healthy adult donors, and leukocyte concentrates were prepared by centrifugation at 4000g/20 min/20°C. PMNLs were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories GmbH, 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 mononuclear leukocytes including lymphocytes and monocytes were gently detached and resuspended in PBS plus 1 mg/ml glucose and 1 mM CaCl₂ (PGC buffer).

**Measurement of Intracellular Ca²⁺ Levels.** MM6 cells or blood monocytes (3 × 10⁶ cells/ml PG buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C. After washing, 3 × 10⁶ cells/ml PG buffer were incubated in a thermally controlled (37°C) fluorometer cuvette in a spectrofluorometer (Amino-Bowman series 2; Thermo Electron Corporation, Waltham, MA) with continuous stirring. Two min prior to stimulation, 1 mM CaCl₂ or 1 mM EDTA was added. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively, and [Ca²⁺]i was calculated according to Grynkiewicz et al. (1985). Fₘₐₓ (maximal fluorescence) was obtained by lysing the cells with 1% Triton X-100 and Fₐ₅₀ by chelating Ca²⁺ with 10 mM EDTA.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting.** Prewarmed (37°C) MM6 cells were preincubated with the indicated concentrations of AKBA or vehicle (DMSO, final concentration ≤1% v/v) for 5 min prior to stimulation with PML (1 μM) for 1 min at 37°C. The reaction was stopped by addition of the same volume of ice-cold 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (SDS-b), samples for SDS-PAGE (alkaloids corresponding to 2 × 10⁶ cells in 20 μl SDS-b) were prepared, and proteins were separated as described (Wertz et al., 2002). Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. Western blotting using phosphospecific antibodies (1:1000 dilution, each; New England Biolabs, Beverly, MA) against pERK1/2 (Thr202/Tyr204) and pp38MAPK (Thr180/Tyr182) was performed using a Li-Cor Odyssey two-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 antibody (Wertz et al., 2002).

**Determination of IP₃ Formation.** Prewarmed (37°C) MM6 cells (1.2 × 10⁶/ml PGC buffer) were either preincubated with the indicated compounds or vehicle, DMSO ≤ 1% (v/v) 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 volumes of ice-cold 1:1000 dilution, each; New England Biolabs, Beverly, MA) against pERK1/2 (Thr202/Tyr204) and pp38MAPK (Thr180/Tyr182) was performed using a Li-Cor Odyssey two-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 antibody (Wertz et al., 2002).
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 PMNLs or in the granulocytic cell line HL-60 (Altmann et al., 2002, 2004). Accordingly, 30 μM AKBA induced a rapid and pronounced elevation of [Ca\(^{2+}\)]\(_i\), in PMNLs, 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 min after exposure to AKBA (see below). In a previous study using MM6 cells (Feist 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 PMNLs, 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 (data not shown).

In analogy to PMNLs, 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. 1, A and C). Finally, no effect was detectable for βBA (30 μM).

AKBA Decreases Elevated [Ca\(^{2+}\)]\(_i\) in PAF-Activated MM6 Cells. To evaluate whether AKBA also affects elevated [Ca\(^{2+}\)]\(_i\) in MM6 cells, MM6 cells were first treated with 100 nM PAF that raises [Ca\(^{2+}\)]\(_i\), and AKBA, AβBA, or U-73122 was added 50 s later. Addition of AKBA or U-73122 evoked an immediate drop of [Ca\(^{2+}\)]\(_i\) (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 to 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 with Fig. 1C). Thus, AKBA decreases $[Ca^{2+}]_i$ in MM6 cells to a comparable extent as found for cells exposed only to AKBA (73 ± 7 nM) or U-73122 (88 ± 8 nM), respectively (Fig. 2, compare with Fig. 1C). Thus, AKBA decreases $[Ca^{2+}]_i$ in MM6 cells to a comparable extent as found for cells exposed only to AKBA (73 ± 7 nM) or U-73122 (88 ± 8 nM), respectively (Fig. 2, compare with Fig. 1C).

AKBA Attenuates Agonist-Induced Elevation of $[Ca^{2+}]_i$. We sought to investigate whether AKBA could also prevent agonist-induced elevations of $[Ca^{2+}]_i$. Agents that elevate $[Ca^{2+}]_i$, involving PLC/IP3 signaling (e.g., PAF, fMLP, and m-3M3FBS) but also stimuli that raise $[Ca^{2+}]_i$, independent of the PLC/IP3 pathway like ionomycin or thapsigargin (TG) were added to MM6 cells that received BAs or U-73122, 20 s prior to agonist addition. As shown in Fig. 3A, AKBA and U-73122, but not AβBA, potently inhibited the subsequent $Ca^{2+}$ mobilization 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 to 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 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 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) $Ca^{2+}$ mobilization.

It appeared possible that the suppressive effects of AKBA observed in MM6 could be related to the fact that MM6 is a human leukemia 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 ($\geq 30$ μM) caused a rather slow but continuous increase in $[Ca^{2+}]_i$, seemingly related to cell lysis or unspecific toxic effects of the compound (data not shown).

AKBA Attenuates $Ca^{2+}$ Mobilization 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/IP3-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. 4, A and B, left panel), although slightly higher concentrations of AKBA and U-73122 were required as compared with those needed to suppress total $Ca^{2+}$ mobilization in the presence of extracellular $Ca^{2+}$, Surprisingly, also, $Ca^{2+}$ mobilization from internal storage sites induced by TG (Fig. 4B, right panel) was partly antagonized by AKBA, implying that PLC inhibition may not be the sole mechanism by which AKBA affects $[Ca^{2+}]_i$, since TG-mediated $Ca^{2+}$ mobilization circumvents the PLC/IP3 route.

AKBA Attenuates fMLP-Evoked MAPK Activation. Cell activation, reflected by elevations in $[Ca^{2+}]_i$, is often accompanied by induction of signaling pathways leading to enhanced levels of phosphorylated members of the MAPK family (Belcheva and Coscia, 2002). The p38MAPK and ERKs are key effectors in the cellular signaling network of leukocytes. Addition of AKBA to resting MM6 cells caused a reduction of the basal levels of phosphorylated ERK-2, whereas phosphorylation of p38MAPK was not affected (Fig. 5A). Note that the phosphorylation state of MAPK solely indicates the activation of the kinases but might not exactly reflect the protein kinase activity toward its substrate(s).

Next, we examined whether AKBA prevents the activation of p38MAPK and ERKs induced by fMLP that, in contrast to PAF, causes prominent phosphorylation of the MAPK in MM6 cells (D. Poockel and O. Werz, unpublished data). Indeed, activation of ERKs was potently prevented by AKBA; again, the amounts of phosphorylated ERK-2 were lower after preincubation with AKBA (10 and 30 μM) as compared with vehicle-treated control cells (Fig. 5B). AKBA also attenuated fMLP-induced phosphorylation of p38MAPK (Fig. 5B). It was shown that U-73122 substantially reduces the activation of p38MAPK in monocyctic cells stimulated by lysophosphatidylcholine (Jing et al., 2000), and we found significant inhibition of fMLP-induced ERK2 activation by U-73122 in MM6
AKBA and U-73122 antagonize agonist-induced Ca\textsuperscript{2+} mobilization. MM6 cells were prepared as described in Fig. 1. A, cells were treated with vehicle (v), AKBA (3, 10, and 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\textsuperscript{2+}]\textsubscript{i}, was determined. Values are given as mean ± S.E., n = 4 to 5, and compared with the positive controls, \( p < 0.05 \) (+) or \( p < 0.01 \) (++). B, original Ca\textsuperscript{2+} recordings of measurements conducted for Fig. 3A. Left, cells were pretreated with vehicle (veh), AKBA (3, 10, and 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, 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 three independent determinations. C, efficacy of AKBA and U-73122 to inhibit agonist-induced Ca\textsuperscript{2+} mobilization depends on the preincubation period. Cells were incubated with AKBA (30 μM) or U-73122 (3 μM) for 20 s or 20 min. Then, PAF or fMLP (0.1 μM each) was added as indicated either 20 s (black bars) or 20 min (white bars) after AKBA or U-73122. The resulting maximum increase in [Ca\textsuperscript{2+}]\textsubscript{i}, was determined and compared with vehicle-treated controls, given as percentage of control ± S.E., n = 3 to 5. Statistical analysis was performed prior to normalization, \( p < 0.05 \) (+) or \( p < 0.01 \) (++). D, Fura-2-loaded primary monocytes (3 × 10\textsuperscript{5}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 three independent determinations.

### Table 1

AKBA but not U-73122 causes sustained depression of [Ca\textsuperscript{2+}]\textsubscript{i}.

MM6 cells in PGC buffer were preincubated with vehicle (DMSO), AKBA, AβBA, or U-73122 for 20 s or 20 min, and [Ca\textsuperscript{2+}]\textsubscript{i} was determined, given as mean ± S.E., n = 4 to 6, \( p < 0.01 \) (**).

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<th>Agonist</th>
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<td>AβBA (30 μM)</td>
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<td>U-73122 (3 μM)</td>
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Cells (data not shown). AβBA, however, was inactive (data not shown).

### Effects of Boswellic Acids on Cellular PLC Activity.

To test whether AKBA (in analogy to U-73122) inhibits cellular PLC activity, we assayed the effects of AKBA on the IP\textsubscript{3} formation in intact MM6 cells. Cellular PLC was directly activated using 100 μM m-3M3FBS to obtain a prominent increase in IP\textsubscript{3} production (7.9-fold elevation, Fig. 6B). In agreement with its ability to block total Ca\textsuperscript{2+} mobilization, U-73122 (5 μM) inhibited m-3M3FBS-induced IP\textsubscript{3} formation (~80%, Fig. 6A). In contrast to its ability to decrease [Ca\textsuperscript{2+}]\textsubscript{i}, AKBA failed to significantly suppress IP\textsubscript{3} formation. Intriguingly, AβBA, which hardly affected Ca\textsuperscript{2+} homeostasis, inhibited m-3M3FBS-induced IP\textsubscript{3} generation (~50%, Fig. 6A).

We then examined whether AKBA, AβBA, or U-73122 could also affect the basal IP\textsubscript{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\textsubscript{3} formation as compared with vehicle-treated control
cells (Fig. 6B). Possibly, this elevation of IP₃ 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 IP₃ production. Thus, a correlation between the suppression of Ca²⁺ homeostasis and inhibition of PLC by AKBA is not readily apparent, suggesting that AKBA operates at alternate targets than PLC to impair [Ca²⁺]ᵢ.

### Inhibitors of Plasma Membrane Ca²⁺ Channels Mimic the Effects of AKBA and Abolish AKBA-Induced Decrease of [Ca²⁺]ᵢ

The fact that AKBA attenuates the secondary phase of TG-induced Ca²⁺ mobilization, which represents Ca²⁺ influx from the extracellular space, prompted us to elucidate if the AKBA-induced loss of intracellular (cytoplasmic) Ca²⁺ may be due to inhibition of plasma membrane Ca²⁺ influx channels such as store-operated Ca²⁺ channels (SOCCs), nonselective cation channels (NSCCs), or voltage-gated Ca²⁺ channels. SK&F96365, an inhibitor of NSCC and SOCC, reduced basal [Ca²⁺]ᵢ in MM6 cells and was able to prevent the subsequent decrease of [Ca²⁺]ᵢ induced by AKBA (Fig. 7). To distinguish between NSCC and SOCC, we applied 2-APB (50 μM), which blocks SOCCs and, on the other hand, LOE908 (10 μM), which selectively inhibits NSCC. Both 2-APB and LOE908 decreased basal [Ca²⁺]ᵢ and prevented the effects of AKBA (Fig. 7). In contrast, inhibitors of voltage-gated Ca²⁺ channels (300 nM α-conotoxin MVIIA or 1 μM verapamil, which block L- or N-type channels, respectively), SR-Ca²⁺ release channels (10 μM neomycin), or blockers of the Na⁺-Ca²⁺ exchanger (SEA0400 or KB-R7943, 10 μM each) failed to significantly decrease basal [Ca²⁺]ᵢ and to prevent effects of AKBA (data not shown). Together, these results indicate that AKBA may mediate the decrease of [Ca²⁺]ᵢ by blocking Ca²⁺ influx from the extracellular space via inhibition of SOCC/NSCC.

### Discussion

AKBA and KBA were shown to induce Ca²⁺ mobilization and activation of MAPK in primary PMNLs and granulocytic HL-60 cells, involving pertussis toxin-sensitive proximal signaling pathways (Altmann et al., 2002, 2004). Activation of these central signaling events were linked to typical functional responses of granulocytes, including peroxide formation and enhanced metabolism of arachidonic acid; 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. (2005) showed that in activated human monocytes, BAs down-regulate TNFα expression via a direct inhibition of IxB kinases, providing a molecular basis for the anti-inflammatory properties of BAs. The result from the present investigation focusing on central signaling pathways in monocytes provides additional evidence for an anti-inflammatory implication of AKBA at the cellular level. Thus, AKBA decreased the basal [Ca²⁺]ᵢ, prevented agonist-
Fig. 6. Effects of BAs on IP₃ formation. A, prewarmed MM6 cells (1.2 × 10⁶/ml PGC buffer) were treated with vehicle (DMSO), AKBA (30 μM), AjBA (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 volumes of ice-cold HClO₄ (20%, v/v), and extraction and evaluation of IP₃ released were determined according to the manufacturer’s instructions (IP₃ [³H] Bistriak Assay System; GE Healthcare). Data are expressed as percentage of vehicle-treated (unstimulated) control ± S.E., n = 3 to 4. Statistical analysis was performed prior to normalization, p < 0.05 (⁎) or <0.01 (⁎⁎). B, prewarmed MM6 cells (1.2 × 10⁶/ml PGC buffer) were incubated with vehicle (DMSO), AKBA (3, 10, and 30 μM), AjBA (30 μM), U-73122 (5 μM), or m-3M3FBS (100 μM) for 15 s. Incubations were stopped by the addition of 0.2 volumes of ice-cold HClO₄ (20%, v/v). The subsequent extraction procedure is identical to the description in A. Data are expressed as percentage of vehicle-treated (unstimulated) control ± S.E., n = 3 to 4. Statistical analysis was performed prior to normalization, p < 0.05 (⁎) or <0.01 (⁎⁎).

Fig. 7. Effects of plasma membrane Ca²⁺ channel inhibitors on Ca²⁺ homeostasis and on the actions of AKBA. Fura-2-loaded MM6 cells (3 × 10⁶/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 CaCl₂. Then, cells were stimulated with AKBA (30 μM), and [Ca²⁺]ᵢ, was determined. Traces are representative for three to four independent determinations.

induced Ca²⁺ mobilization, and blocked the activation of ERK1/2 and p38 MAPK, signaling 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 Ca²⁺ (Pollaud-Cherion et al., 1998; See et al., 2004), providing a possible link between interference with Ca²⁺ and down-regulation of NF-κB and TNFα.

Apparently, in view of the opposite, agonistic effects on PMNLs 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 signaling events, implying anti-inflammatory functionality. PMNLs 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 Ca²⁺ homeostasis in analogy to AKBA are obvious in leukocytes exposed to arachidonic acid that decreases [Ca²⁺]ᵢ in peritoneal macrophages (Randriamampita and Trautmann, 1990) and in MM6 cells (D. Poeckel and O. Werz, unpublished data) but on the other hand evokes Ca²⁺ mobilization in PMNLs (Naccache et al., 1989).

Many effector enzymes like phospholipases, 5-LO, and protein kinases respond to and are regulated by an elevation of [Ca²⁺]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; 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), topoisomerases I and II (Syrovets et al., 2000), as well as iKB 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 [Ca²⁺]ᵢ of resting cells but also caused an immediate drop of the elevated [Ca²⁺]ᵢ, after challenge with PAF, displaying comparable kinetics. Moreover, both agents reduced agonist-evoked Ca²⁺ mobilization, which in fact is a characteristic for monocyte activation by external stimuli (Kim et al., 1992; Bernardo et al., 1997; Li et al., 2002). Such Ca²⁺-antagonizing 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 Ca²⁺ fluxes induced by the ER/SR-Ca²⁺-ATPase inhibitor TG or by the Ca²⁺-ionophore ionomycin, which both
circumvent PLC/IP$_3$ for Ca$^{2+}$ mobilization (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 IP$_3$ receptor could be a plausible explanation. Surprisingly, however, AKBA significantly inhibited TG-induced Ca$^{2+}$ mobilization from internal stores and also the sustained elevation of [Ca$^{2+}$], 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 IP$_3$. An important finding that favors a PLC-independent mechanism is the failure of AKBA to efficiently suppress the release of intracellular IP$_3$. Strikingly, in contrast to AKBA, ABBA clearly failed to counteract m-3M3FBS-induced Ca$^{2+}$ mobilization, 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$^{2+}$], induced by AKBA. This hypothesis is further supported by the fact that AKBA on one hand even slightly increased basal IP$_3$ levels about 2-fold, which should actually lead to Ca$^{2+}$ release from internal storage sites. However, in contrast, there is a strong decrease in the basal [Ca$^{2+}$], 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$^{2+}$], observed with AKBA and were able to inhibit the subsequent decrease of [Ca$^{2+}$], induced by AKBA. Of interest, also for U-73122, inhibition of plasma membrane Ca$^{2+}$ channels has been accounted for reduced [Ca$^{2+}$], (see Feist 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 toward predominant extrusion that, as a result, leads to impaired [Ca$^{2+}$]. However, more detailed experiments are required 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 antagonizing Ca$^{2+}$, AKBA potently prevented fMLP-induced activation of p38MAPK 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). p38MAPK inhibitors have been developed to treat for example rheumatoid arthritis (Pargellis and Regan, 2003) and Crohn’s disease (Hommes et al., 2002), inflammatory disorders that 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 p38MAPK activation in monocytic THP-1 cells (Jing et al., 2000).

In summary, our data show that AKBA is capable to suppress central signaling 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 properties of BAs.

References
Boswellic acids activate p42(MAPK) and p38 MAPK and stimulate Ca$^{2+}$ mobilization. Biochem Biophys Res Commun 290:185–190.


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