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Boswellic Acids Activate p42^{MAPK} and p38 MAPK and Stimulate Ca^{2+} Mobilization[1]

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Here we show that extracts of *Boswellia serrata* gum resins and its constituents, the boswellic acids (BAs), activate the mitogen-activated protein kinases (MAPK) p42^{MAPK} and p38 in isolated human polymorphonuclear leukocytes (PMNL). MAPK activation was rapid and transient with maximal activation after 1–2.5 min of exposure and occurred in a dose-dependent manner. The keto-BAs (11-keto-β-BA and 3-*O*-acetyl-11-β-keto-BA) gave substantial kinase activation at 30 μM, whereas other BAs lacking the 11-keto group were less effective. Moreover, 11-keto-BAs induced rapid and prominent mobilization of free Ca^{2+} in PMNL. Inhibitor studies revealed that phosphatidylinositol 3-kinase (PI 3-K) is involved in BA-induced MAPK activation, whereas a minor role was apparent for protein kinase C. MAPK activation by 3-*O*-acetyl-11-β-keto-BA was partially inhibited when Ca^{2+} was removed by chelation. Our results suggest that 11-keto-BAs might function as potent activators of PMNL by stimulation of MAPK and mobilization of intracellular Ca^{2+}.

Key Words: **boswellic acids; polymorphonuclear leukocyte; p38 MAPK; p44/42^{MAPK}; Ca^{2+}; phosphatidylinositol 3-kinase; protein kinase C.**

Abbreviations used: AB, antibody; A-β-BA, 3-*O*-acetyl-β-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-β-boswellic acid; KBA, 11-keto-β-boswellic acid; β-BA, β-boswellic acid; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; JNK, c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; PAF, platelet-activating factor; PBS, phosphate-buffered saline, pH 7.4; PG buffer, PBS containing 1 mg/ml glucose; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$; PI 3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMNL, polymorphonuclear leukocytes; SDS-b, 2× SDS–PAGE sample loading buffer; WB, Western blotting.

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Boswellic acids (BAs) are pentacyclic triterpenes that have been identified as the active principles of Frankincense, the gum resin of *Boswellia* species. BA derivatives inhibit human leukocyte elastase *in vitro* (1) and suppress the biosynthesis of proinflammatory leukotrienes by direct inhibition of leukocyte 5-lipoxygenase (2), which could explain the anti-inflammatory properties of Frankincense stated in several experimental animal models and clinical trials, for review see (3). In addition, BAs inhibit topoisomerases in myeloid cells and have been shown to induce differentiation and apoptosis in leukemia cell lines (4–6) as well as apoptosis in malignant glioma cells (7). However, little is known about the effects of BAs on biochemical and cellular signaling pathways in leukocytes.

Transduction of extracellular signals leading to a diverse array of cellular responses includes the activation of specific kinases. The mammalian mitogen-activated protein kinase (MAPK) superfamily consists of at least four distinct groups, organized in signaling modules (MEKK/MEK/MAPK) that transmit extracellular signals by sequential phosphorylation and activation of the components of a respective cascade: the extracellular signal-regulated kinases 1 and 2 (ERK1/2, also termed p44/42^{MAPK}), the p38 MAPKs, the c-Jun NH_2 terminal kinases (JNKs), and the Big MAPK 1 (also termed ERK5) (8, 9). Although structurally related, these kinases can act on different molecular substrates (transcription factors, protein kinases) and their actions may lead to distinct and sometimes opposite biological functions (10). Whereas p44/42^{MAPK} are mainly activated in response to mitogenic stimuli, such as growth factors and G-protein-coupled receptor agonists, p38 MAPK, JNKs and ERK5 are activated in response to various forms of cell stress or cytokines (8, 11). The p44/42^{MAPK} pathway may play a pivotal role in cell growth, differentiation and cellular transformation, but also can regulate cellular events, such as secretion and cell motility (10). In neutrophils, the p44/42^{MAPK} pathway has been proposed to play a certain role in neutrophil functions, in response to appropriate external stimuli (12–14). p38 MAPK activation

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results in the production of TNFα and IL-1 and has been implicated in granulocyte apoptosis, adhesion, degranulation, chemotaxis and oxidative burst [reviewed in (15)]. In this study we addressed the effects of BAs on various signaling pathways important for cellular responses of neutrophils, particularly the activation of MAPK pathways and the mobilization of Ca^{2+}.

MATERIALS AND METHODS

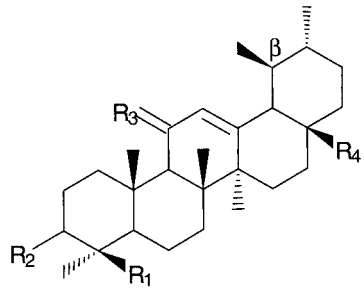
Materials. Ethanolic extracts of *B. serrata* were obtained from Engelhard Arzneimittel GmbH (Niederdorfelden, Germany); A-β-BA, β-BA, AKBA, and KBA were purchased from ChromaDex (Laguna Hills, CA). α-Amyrin and ursolic acid were from Extrasynthèse (Genay, France). Activated (rat, recombinant) $p42^{MAPK}$ isoform was from Biomol; [γ-^{32}P]ATP (110 TBq/mmol) was purchased from Amersham–Pharmacia Biotech (Freiburg, Germany). Materials and reagents: Nycoprep, PAA Laboratories (Linz, Austria); Ca^{2+}-ionophore A23187, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), and myelin basic protein (MBP) were from Sigma (Deisenhofen, Germany). BAPTA/AM and Fura-2/AM were from Calbiochem (Bad Soden, Germany). RO-31-8425 was from Alexis, Switzerland, and GF109203x and wortmannin, from Biotrend (Colonia, Germany).

Cells. Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation for preparation of leukocyte concentrates. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously (16). PMNL (5×10^6 cells/ml; purity > 96–97%) were finally resuspended in PBS plus 1 mg/ml glucose (PG buffer), or alternatively in PBS plus 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated.

Measurement of intracellular Ca^{2+} levels. Freshly isolated PMNL (1×10^7 in 1 ml PGC buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C. Cells were washed, resuspended in 1 ml PGC buffer and transferred into a thermally controlled (37°C) fluorometer cuvette in a spectrofluorometer (Aminco–Bowman Series 2) 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.* (17). 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.

MAPK activation. Freshly isolated PMNL (5×10^6) were resuspended in PGC buffer or in PG buffer containing 1 mM EDTA and/or 30 μM BAPTA/AM, 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× SDS–PAGE sample loading buffer [SDS-b: 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol], vortexed, and heated for 6 min at 95°C. Twenty microliters of total cell lysates was analyzed for activated MAPK by SDS–PAGE and Western blotting (WB) or by in-gel kinase assay.

SDS–PAGE and Western blotting. Total cell lysates (20 μl) were mixed with 4 μl of glycerol/0.1% bromophenol blue (1:1, v/v) and analyzed by SDS–PAGE on a 10% gel. After electroblot to nitrocellulose membrane (Amersham–Pharmacia), blocking with 5% nonfat dry milk for 1 h at RT, membranes were washed and incubated with primary antibody for overnight at 4°C. Phospho-specific antibodies (AB) recognizing $p44/42^{MAPK}$ (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182), and JNK (Thr183/Tyr185) were obtained from New England Biolabs, Inc., and used as 1:2000 dilution. The membranes were washed and incubated with 1:1000 dilution of alkaline phosphatase conjugated IgGs (Sigma) for 2 h at RT. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).



R_1=COOH	R_2= α-OAc	R_3=O	R_4=H	AKBA
R_1=COOH	R_2= α-OH	R_3=O	R_4=H	KBA
R_1=COOH	R_2= α-OAc	R_3=H_2	R_4=H	Acetyl-β-BA
R_1=COOH	R_2= α-OH	R_3=H_2	R_4=H	β-BA
R_1=H	R_2= β-OH	R_3=H_2	R_4=H	α-Amyrin
R_1=H	R_2= β-OH	R_3=H_2	R_4=COOH	β-BA

FIG. 1. Structures of boswellic acids and derivatives.

In-gel kinase assay. Total cell lysates of PMNL corresponding to 0.5×10^6 cells were analyzed for $p42^{MAPK}$ activity by in-gel kinase assay using MBP (0.5 mg/ml) as substrate as described (18). Phosphorylated proteins were visualized using a Fuji Phosphorimager FLA-3000.

RESULTS

Boswellic Acids Activate p38 MAPK and $p42^{MAPK}$ in Human Isolated PMNL

Ethanolic extracts of *B. serrata* resin as well as the four major pentacyclic triterpenic acids present, namely β-boswellic acid (β-BA), 3-*O*-acetyl-β-boswellic acid (A-β-BA), 11-keto-β-boswellic acid (KBA), and 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) (Fig. 1), were assayed for activation of MAPK in freshly isolated PMNL from human peripheral blood. Ca^{2+}-ionophore A23187 was utilized as positive control (18). After stimulation, cells were lysed and total cell lysates were subjected to WB using phospho-specific AB against $p44/42^{MAPK}$, p38 MAPK, and JNKs. In addition, MAPK activities of total cell lysates were assessed by in-gel kinase assay using MBP as substrate.

As can be seen from Fig. 2A, exposure of PMNL to crude extracts of *B. serrata* (0.38 to 13 μg/ml) for 3 min led to a dose-dependent activation of $p42^{MAPK}$ and p38 MAPK, whereas $p44^{MAPK}$ and JNKs (not shown) seemed not to be activated. Similarly, KBA and AKBA (30 μM each, corresponding to $\approx$3.8 μg/ml *B. serrata* extracts) caused activation of $p42^{MAPK}$ and p38 MAPK 1.5 min after addition, whereas β-BA and A-β-BA (30 μM for 1.5 min, each) did not activate $p42^{MAPK}$ and caused only slight activation of p38 MAPK (Fig. 2B). AKBA also slightly activated $p44^{MAPK}$ and was more efficient in

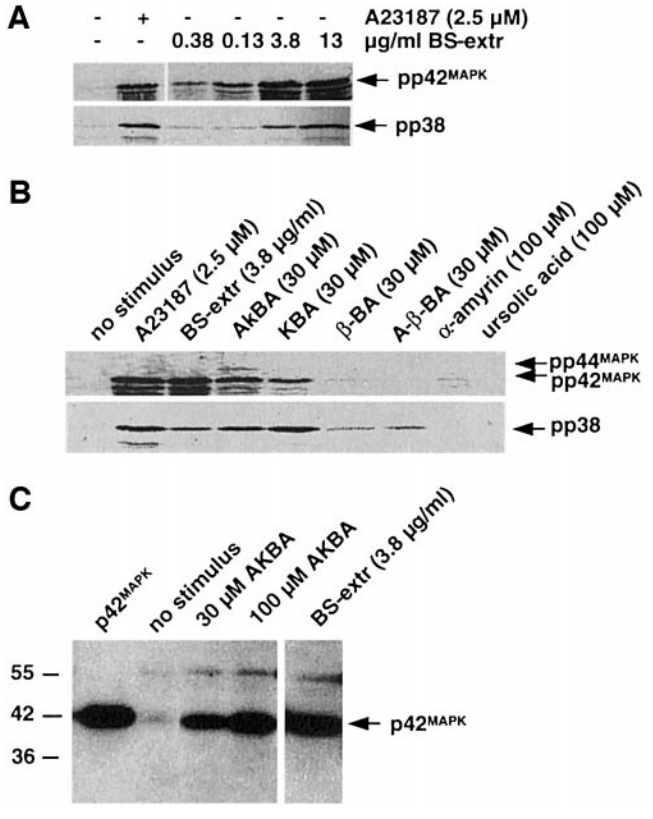


FIG. 2. Activation of MAPK by extracts of *B. serrata* and isolated BAs. To determine activation of p38 MAPK and p44/42^{MAPK}, freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were stimulated with the indicated amounts of *B. serrata* extracts (BS-extr) for 3 min at 37°C (A) or with BS-extr., and the compounds indicated in the figure (B) for 1.5 min at 37°C. After addition of the same volume of ice-cold SDS-b, samples were analyzed for dually phosphorylated p38 MAPK (pp38) or p44/42^{MAPK} (pp44/42^{MAPK}) by WB. (C) Samples from above were assayed for p42^{MAPK} activity by in-gel kinase assay using MBP (0.5 mg/ml) as substrate. Purified recombinant active 42^{MAPK} (10 mU) was used as positive control. The position of p42^{MAPK} (42 kDa) in the gel is indicated. Results are representative of at least three separate experiments.

p42^{MAPK} activation than KBA. However, KBA was virtually equally effective as AKBA in activation of p38 MAPK. The pentacyclic triterpenes α-amyrin and ursolic acid up to 100 μM gave no MAPK activation (Fig. 2B).

Activation of p42^{MAPK} by BAs was confirmed by in-gel kinase assays. Thus in agreement with the results from above (Fig. 2A), cell stimulation by extracts of *B. serrata* resin (3.8 μg/ml) and by AKBA (30 and 100 μM) led to prominent kinase activity (Fig. 2C). Kinase activities were rather low for cells treated with KBA, β-BA and A-β-BA (100 μM each, not shown).

The dose responses for MAPK activation by KBA and AKBA were determined. Both, p42^{MAPK} and p38 MAPK became clearly activated in cells treated with 30 μM AKBA for 1.5 min (Fig. 3A). In contrast, 100 μM KBA was needed for a clear activation of p42^{MAPK}. However, 10 to 30 μM KBA gave substantial activation of p38 MAPK (Fig. 3A). Again, for β-BA and A-β-BA (100 μM each) only weak MAPK activation was obtained (not shown), indicating that the 11-keto group is a structural requirement for substantial activation of MAPKs. Activation of both p42^{MAPK} and p38 MAPK by BAs was rapid and occurred within 30 s, peaking around 1 to 2.5 min and declining after about 10 min (Fig. 3B). In this respect, no appreciable differences between the various BAs were observed.

Boswellic Acids Induce Ca^{2+} Mobilization in PMNL

We considered the possibility that BAs could induce Ca^{2+} mobilization in PMNL, one of the hallmarks of PMNL activation in response to various agonists. As shown in Fig. 4, exposure of freshly isolated PMNL to AKBA or KBA (30 μM, each) caused a rapid (within 10 to 15 s) and prominent elevation of intracellular Ca^{2+}, that was comparable to the effect of chemotactic fMLP, used as a positive control. Thus, intracellular Ca^{2+} levels of unstimulated cells were about 40–50 nM, rising to about 240 nM upon stimulation with 1 μM fMLP. Upon exposure to 10 and 30 μM AKBA, intracellular Ca^{2+} increased to about 120 and 230 nM, respectively. Similarly, 10 and 30 μM KBA raised intracellular Ca^{2+} to 105 and 145 nM. In contrast, β-BA, A-β-BA, α-amyrin or ursolic acid (30 μM each) were virtually not effective (not shown).

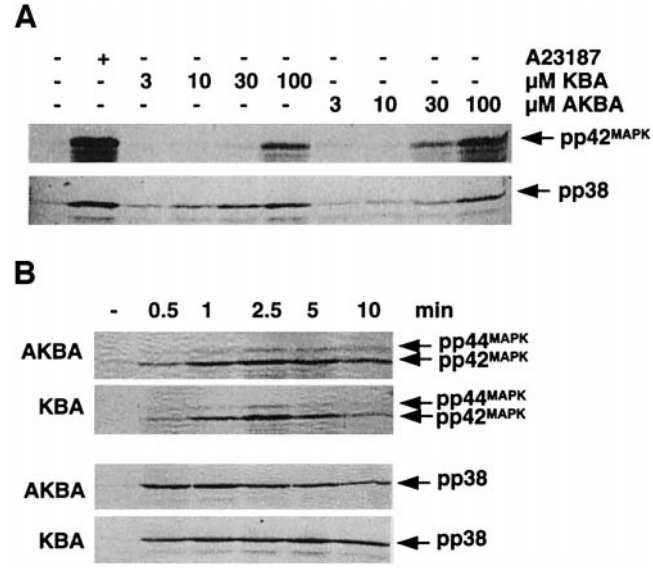


FIG. 3. Dose response and time course of MAPK activation by AKBA and KBA. (A) Dose response. Freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were stimulated with the indicated amounts of AKBA or KBA for 1.5 min at 37°C, ionophore A23187 (2.5 μM) was used as positive control. (B) Time course. Freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were stimulated with 30 μM AKBA or 100 μM KBA for the indicated times at 37°C. All incubations were terminated by addition of the same volume of ice-cold SDS-b and analyzed for dually phosphorylated p38 MAPK or p44/42^{MAPK} by WB.

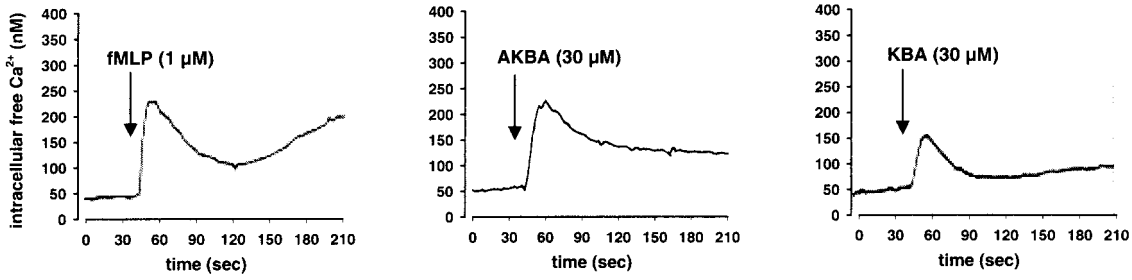


FIG. 4. BAs induce the mobilization of Ca^{2+}. To Fura-2-loaded PMNL (1×10^7/ml PGC buffer) the indicated stimuli were added and the fluorescence was measured. Intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment of three or four.

Role of Ca^{2+}, PKC, and PI 3-K in AKBA-Induced MAPK Activation

We attempted to elucidate the upstream signaling pathways leading to activation of p38 MAPK and $p42^{MAPK}$ induced by AKBA. Activation of MAPK by various agonists [fMLP, platelet-activating factor (PAF)] was shown to depend in part on Ca^{2+}. Removal of extracellular Ca^{2+} by EDTA and/or depletion of intracellular Ca^{2+} by cell-permeable BAPTA/AM only partially suppressed AKBA-induced activation of $p42^{MAPK}$ (Fig. 5A). Thus Ca^{2+} seems to play a minor role in AKBA-induced MAPK activation.

Next, the involvement of protein kinase C (PKC) and PI 3-K in MAPK activation was investigated. As depicted from Fig. 5B (WB) and C (in-gel kinase assay), inhibition of PKC by RO-31-8425 (1 μM) slightly attenuated activation of $p42^{MAPK}$ and p38 MAPK induced by 30 μM AKBA, whereas GF109203x (1 μM) rather enhanced $p42^{MAPK}$ activation. Thus particular isoforms of PKC (affected by RO-31-8425) could be partially involved in activation of $p42^{MAPK}$ and p38 MAPK. Wortmannin, an inhibitor of PI 3-K, was used to determine a possible participation of PI 3-K in the upstream activation of MAPKs. As shown in Figs. 5B and 5C, wortmannin strongly attenuated $p42^{MAPK}$ activation evoked by 30 μM AKBA and reduced p38 MAPK activation, indicating an involvement of PI 3-K in the upstream cascades required for AKBA-induced MAPK signaling. Similar results were obtained when KBA (100 μM) was used as stimulus (not shown).

DISCUSSION

Here we demonstrate that BAs the major constituents of extracts of *B. serrata* gum can stimulate important cellular signaling pathways in isolated human PMNL. Thus, 11-keto-BAs caused substantial activation of $p42^{MAPK}$ and p38 MAPK, and induced the mobilization of intracellular Ca^{2+}. Recently, the tetracyclic triterpene 3-oxo-tirucallic acid was identified in *B. serrata* extracts as a component that enhanced 5-LO product formation, accompanied with moderate Ca^{2+} mobilization, and that increased phosphorylation of MEK-1/2 (19).

Activation of $p42^{MAPK}$ and p38 MAPK was determined by WB using specific AB that detect only the dually phosphorylated (active) form of the kinases, and was confirmed by in-gel kinase assays using MBP as substrate, a suitable method to detect $p44/42^{MAPK}$ activities in PMNL extracts (13). Activation of both MAPKs by BAs was rapid and transient, and concentrations of 10 to 100 μM of AKBA or KBA necessary to mobilize Ca^{2+} or to activate MAPKs, are in the range of the concentrations required for various biological effects observed by others (1, 4). The presence of the 11-keto-group appears important for the effects of BAs, since β-BA and A-β-BA were much less efficient than AKBA or KBA in MAPK activation, and only AKBA and KBA caused Ca^{2+} mobilization in PMNL. Notably, the keto group was also required for efficient inhibition of agonist-induced leukotriene formation in neutrophils (20) and for pronounced inhibitory effects on DNA, RNA and protein synthesis in HL60 cells (21). Although no receptors for BAs have been identified yet, it is possible that 11-keto-BAs may bind a certain cell surface receptor transducing the signal to the respective cellular target(s). The 11-keto group might be a structural determinant for interaction with such a putative receptor(s).

The effects of BAs elicited in PMNL in this study resemble those evoked by chemotactic factors. Thus, similar as 11-keto-BAs, fMLP and PAF induced the activation of $p42^{MAPK}$ and p38 MAPK [but not of JNKs (22)] and caused rapid mobilization of intracellular Ca^{2+} in PMNL (23–26). The characteristics of fMLP-induced MAPK activation, such as the time course, the partial dependence on Ca^{2+} as well as on PKC, and the involvement of PI 3-K, particularly in the activation of 42^{MAPK} (22, 25, 27, 28), are conform with those induced by AKBA or KBA. Therefore, BAs might share signal transduction pathways with fMLP in PMNL. G-proteins couple the fMLP-receptor interaction with subsequent biochemical and functional responses (29) and it was concluded that fMLP-induced activation of MAPK is a consequence of ligation of the

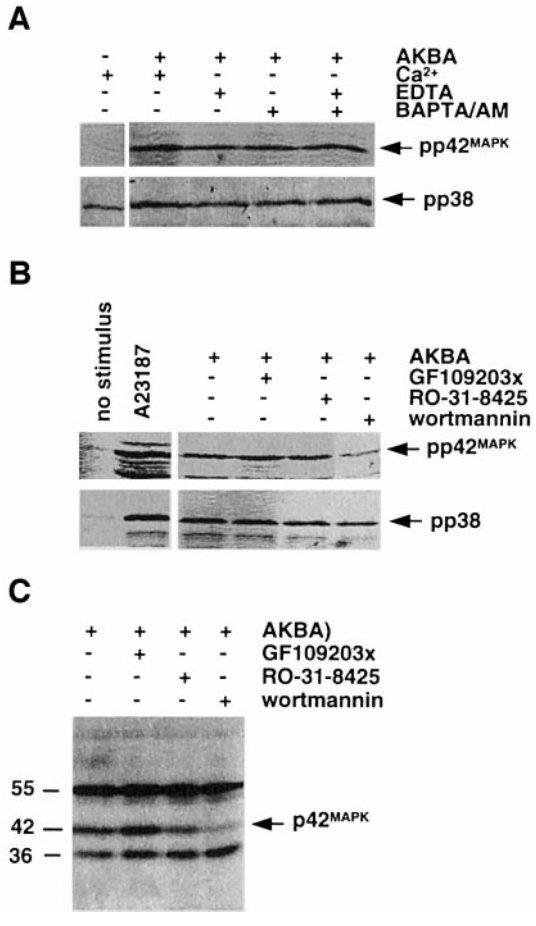


FIG. 5. Effects of Ca^{2+}-depletion, PKC and PI 3-K inhibitors on MAPK activation induced by AKBA. (A) Effects of Ca^{2+} chelation. $CaCl_2$ (1 mM), EDTA (1 mM), and BAPTA/AM (30 μM) were added to 5×10^6 freshly isolated PMNL in PG buffer as indicated. After 15 min at 37°C, 30 μM AKBA was added and the incubations were continued for another 1.5 min. Then, incubations were terminated by addition of the same volume of ice-cold SDS-b. (B, C) Effects of PKC and PI 3-K inhibition. GF109203x, RO-31-8425 (1 μM each), and wortmannin (0.2 μM) were added to 5×10^6 freshly isolated PMNL in 100 μl PG buffer as indicated. After 15 min at 37°C, AKBA (30 μM) was added and the incubations were continued for another 1.5 min. Then, incubations were terminated by addition of the same volume of ice-cold SDS-b and analyzed for dually phosphorylated p38 MAPK or $p44/42^{MAPK}$ by WB (B) or analyzed for $p42^{MAPK}$ by in-gel kinase assay (C). The position of $p42^{MAPK}$ is indicated, bands at 55 and 36 kDa may result from other unidentified kinases. Results are representative of at least three separate experiments.

fMLP receptor (30). Thus, it is reasonable to speculate that BAs could directly activate G-protein-coupled receptors. Alternatively, BAs might act indirectly by stimulation of mediator release that bind such receptors leading to activation of MAPK. Future work is necessary to elucidate such mechanisms.

PKC isoforms have been implicated as components in the signal transduction cascade resulting in activation of $p44/42^{MAPK}$ as well as of p38 MAPK in PMNL. RO-31-8524 only partially inhibited AKBA-induced MAPK activation, and GF109203x even increased the effects of AKBA for unknown reasons. Similarly, minor importance of PKC was proposed in the upstream regulation of fMLP-induced activation of $p44/42^{MAPK}$ and p38 MAPK in neutrophils (28). In PMNL, rapid activation of the PI 3-K occurs in response to various agonists like fMLP or arachidonic acid (31), which for example can regulate chemotaxis (32). It was shown that in PMNL, PI 3-K is partially involved in agonist-induced $p44/42^{MAPK}$ activation (25, 28), but apparently plays a minor role in the activation of p38 MAPK (28, 33). Wortmannin, a selective inhibitor of PI 3-K, considerably suppressed the AKBA-induced activation of $p42^{MAPK}$ and also reduced p38 MAPK activation (Figs. 5B and 5C). Thus, PI 3-K might be an important component in the signal transduction of $p42^{MAPK}$ activation induced by AKBA.

Rapid elevation of intracellular Ca^{2+} mediates many PMNL responses to receptor agonists (34). Ca^{2+} has been implicated in the regulation of protein phosphorylation (35), and Ca^{2+} selective ionophores (ionomycin, A23187) caused strong activation of $p44/42^{MAPK}$ and p38 MAPK in human neutrophils (36, 37). AKBA and KBA rapidly increased intracellular Ca^{2+} levels in PMNL, with comparable time courses and magnitudes as observed for fMLP (Fig. 4) and this might be the reason for AKBA-induced MAPK activation, as it was observed after stimulation with Ca^{2+} ionophores. In agreement with the results of Ferby *et al.* using PAF as neutrophil agonist (25), depletion of Ca^{2+} reduced the magnitude of AKBA-induced MAPK only partially, indicating that alternative mechanisms than elevated Ca^{2+} may contribute to MAPK activation by BAs.

In summary we could demonstrate, that extracts of *B. serrata* gum and isolated 11-keto-BAs can mobilize intracellular Ca^{2+} and stimulate the activation of $p42^{MAPK}$ and p38 MAPK in PMNL. Since these pivotal signaling events regulate numerous effectors of PMNL, such as superoxide production, phagocytosis of particles, release of lysosomal enzymes, and the release of lipid mediators, it remains a future challenge to investigate whether BAs are capable of stimulating such PMNL functions.

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