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Pharmacologic Modulation of the Bradykinin-Induced Differentiation of Human Lung Fibroblasts: Effects of Budesonide and Formoterol

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Running head: BUD and FM inhibit human lung fibroblast differentiation

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ABSTRACT

Objective. Bradykinin (BK) induces differentiation of lung fibroblasts into myofibroblasts, which play an important role in extracellular matrix remodelling in the airways of asthmatic patients. It is unclear whether this process is affected by anti-asthma therapies. Here, we evaluated whether a glucocorticoid, budesonide (BUD), and a long-acting β_2-agonist, formoterol (FM), either alone or in combination, modified BK-induced lung fibroblast differentiation and which BK-activated intracellular signalling pathways were affected.

Methods. Human foetal lung fibroblasts were incubated with BUD (0.001–0.1 μM) and/or FM (0.0001–0.1 μM) before exposure to BK (0.1 or 1 μM). Fibroblast differentiation into α-smooth-muscle actin-positive ($\alpha-SMA^+$) myofibroblasts, BK2 receptor (B2R) expression, ERK1/2 phosphorylation (p-ERK1/2), intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and p65 NFκB translocation were evaluated.

Results. BUD (0.1 μM) and FM (0.1 μM), alone and in combination, completely inhibited BK-induced α-SMA protein expression and decreased the numbers of α-SMA$^+$ fibroblasts, with a clear reduction in α-SMA stress fibres organization. BUD also completely inhibited the increase of B2R while FM with or without BUD had no effect. BK-induced increases of $[Ca^{2+}]_i$ and p-ERK1/2 were significantly reduced to similar levels by BUD and FM, alone and in combination, whereas p65 translocation was completely inhibited by all treatments.

Conclusion. Both BUD and FM, either alone or in combination, effectively inhibited the BK-induced differentiation of fibroblasts into $\alpha-SMA^+$ myofibroblasts and the intracellular signalling pathways involved in fibroblast activation. These results suggest that BUD and FM therapy has potential to inhibit fibroblast-dependent matrix remodelling in the airways of asthmatic patients.

INTRODUCTION

Bradykinin (BK), a peptide formed from kininogen precursors following the activation of plasma and tissue kallikreins, is known to mediate multiple pro-inflammatory effects in a variety of disorders (1, 2). Most of the biological actions of BK are mediated via an interaction of the B2 constitutive receptor (B2R), which is widely distributed throughout the human body, while the expression of the inducible B1 receptor (B1R) requires induction by pro-inflammatory cytokines, such as IL-1β, TNF-α, or by infectious stimuli (2). In asthma, BK, by increasing the cholinergic tone and acting on inflammatory and structural cells, induces bronchial obstruction, vasodilation, plasma extravasation, stimulation of sensory neurons and increases the synthesis and release of nitric oxide, prostaglandins, leukotrienes, cytokines, eicosanoids and neuropeptides (3-5). Interestingly, BK is also capable not only of stimulating human lung fibroblast contractility, cytokine release and proliferation but also of inducing fibroblast differentiation into α–smooth-muscle actin (α–SMA)-positive myofibroblasts through the activation of B2R (6-8). Myofibroblasts are the cells predominantly responsible for increased production and deposition of extracellular matrix molecules in the airways of asthmatic patients, contributing to airway wall thickening (by increasing the thickness of reticular basement membrane) and to the irreversible or only partially reversible airway obstruction that characterizes this disorder (9).

Fibroblast activation and differentiation is regulated in part by epidermal growth factor (EGF)-like factors and the EGF receptor-dependent downstream signaling pathways, which includes phosphorylation of mitogen-activated protein kinases (MAPKs) (10, 11). In addition to MAPKs, the involvement of the transcription factor nuclear factor kappa B (NFκB) and of a transient elevation of intracellular calcium concentration $[Ca^{2+}]_i$ in (myo)fibroblast activation and differentiation have also been described (11, 12). Of note, previous studies showed that BK-induced myofibroblast differentiation is dependent on ERK1/2 pathway and on intracellular calcium mobilization, whilst NFκB is able to modulate B2R expression in fibroblasts (8, 12, 13).

Little information is available as to whether the combination of an inhaled corticosteroid (ICS) and a long-acting β2-agonist (LABA) – currently the most successful asthma therapy (14) – may counteract activation and differentiation of airway fibroblasts in asthma. However, it was shown recently that the combination therapy of the ICS budesonide and the LABA formoterol significantly attenuated the number of submucosal tissue myofibroblasts enhanced after allergen inhalation (15). Furthermore, budesonide/formoterol combination therapy was shown to decrease the thickness of reticular basement membrane and bronchial wall in patients with asthma (16). It is not known what cellular and intracellular pathways are involved in these effects.

The present study was designed to evaluate *in vitro* whether budesonide and formoterol, either alone or in combination, could modify the BK-induced differentiation of fibroblasts into α-SMA-positive myofibroblasts. In addition, the effects of the two drugs were investigated on the BK-induced B2R expression and on the downstream pathways possibly involved in BK-induced fibroblast activation: ERK1/2 phosphorylation (p-ERK1/2), intracellular Ca^{2+} mobilization and p65 NFκB translocation.

MATERIALS AND METHODS

Fibroblast culture

The human foetal lung fibroblast cell-line (HFL-1) was obtained from the American Type Culture Collection (Manassas, VA, USA) and used in all experiments. Cells were cultured in 75-cm^2 tissue culture flask with high-glucose Dulbecco's Modified Eagle medium supplemented with 10% foetal calf serum (FCS) and penicillin/streptomycin (5,000 IU/ml). Fibroblasts were passaged approximately every 7 days after reaching confluence by dissociating the monolayer with 1:1 0.05% trypsin:0.1% ethylenediamine tetraacetate solution (EDTA) and used in passages 2-10. All cells were mycoplasma negative, checked by the Hoechst staining method (17). In all experiments, the vehicle for budesonide dilution was ethanol, whereas for formoterol dimethyl sulfoxide (DMSO) was used. The final concentration of both vehicles was <0.05% and did not affect cell viability as assessed by trypan blue dye exclusion as well as all cell functions tested (data not shown).

Experimental design

The following read-outs were evaluated in HFL-1 fibroblasts: i) fibroblast differentiation into α-SMA positive myofibroblasts; ii) B2R protein expression; iii) ERK1/2 phosphorylation; iv) intracellular calcium mobilization; and v) p65 NFκB translocation. The α-SMA and B2R expression were evaluated after 48 h incubation with/without BK (1.0 μM) in the presence or absence of BUD (0.001–0.1 μM) and/or FM (0.0001–0.1 μM). ERK1/2 MAPK phosphorylation was evaluated in cells pre-incubated for 30 or 60 min with BUD (0.001–0.1 μM) and/or FM (0.001–0.1 μM) and then stimulated for 5 min with BK (0.1 μM). For evaluation of Ca^{2+} mobilization, cells were pre-incubated for 5, 15, 30 or 60 min with BUD (0.1 μM) and/or FM (0.1 μM) before exposure to BK (1.0 μM). For evaluation of p65 NFκB translocation, cells were exposed to BK (1.0 μM) for 180 min with or without BUD (0.1 μM) and/or FM (0.1 μM).

Western blot analysis

Analysis of α-SMA and B2R expression, and of ERK1/2 and NFκB activation, was performed by Western blot. Cellular lysates were prepared as described previously (12, 18, 19). Equal amounts of total protein were resolved on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to PVDF membrane (Immobilon-P Millipore Corporation, Billerica, Massachusetts, USA). The membranes were blocked with 3% nonfat dry milk (α-SMA analysis), 5% BSA (B2R), or 5% nonfat dry milk (ERK analysis) in Tris-buffered saline containing 0.05% Tween 20. Subsequently, membranes were incubated with a mouse anti-α-SMA

antibody (Dako cytomation, Carpinteria, CA, USA), a mouse monoclonal anti-B2R antibody (BD Biosciences Inc., Milan, Italy), rabbit anti-total ERK1/2 and anti-phosphorylated ERK1/2 antibodies (Cell Signalling, Technology®, Beverly, MA, USA). Appropriate peroxidase-conjugated secondary antibodies were used. Then specific reactive proteins were visualized using the enhanced chemiluminescence system (SuperSignal West Dura Extended Duration Substrate, Pierce Inc, USA). The relevant band intensities were quantified using a Versadoc Imaging System model 3000 (Biorad Laboratories, Inc.; Hercules, CA, USA). For α-SMA and B2R expression, to ensure equal protein loading, the membranes were stripped and re-probed with anti-β-actin antibody (clone C4) (Boehringer Mannheim Inc, Mannheim, Germany). To quantify the proportion of activated ERK (pERK) at the different experimental conditions, the ratio between pERK and the total endogenous ERK expression was calculated (20).

For the study of p65 NFκB translocation, proteins from nuclear fractions were prepared using QProteome cell compartment kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Sample protein concentrations were determined by Bradford assay, and the samples were stored at -80°C. Nuclear extract was separated by electrophoresis on SDS-PAGE and transferred to PVDF. Membranes were blocked in 5% milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with a rabbit polyclonal anti-NKkB p65 antibody (SC-372, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with peroxidase-conjugated secondary antibody, the bands were visualized by the enhanced chemiluminescence system and relevant band intensities were quantified using a Versadoc Imaging System model 3000. To ensure equal loading, the membranes were stripped and re-probed with lamin A/C antibody (BD Biosciences, Milan, Italy) for nuclear extracts (21).

Immunostaining of α-SMA

Organization of α-SMA was evaluated by immunofluorescence using a conventional indirect technique (12). Briefly, fibroblasts were cultured on glass cover slips (0.13×10^6 cells/glass cover slip) and stimulated as described earlier. After incubation, cells were fixed in methanol for 5 min at -20°C and then stained with mouse monoclonal antibody against human α-SMA (Sigma-Aldrich S.r.l., Milan, Italy) or with isotype-matched control mouse IgG (Immunotech, Westbrook, ME, USA). FITC-conjugate goat anti-mouse IgG (heavy and light chain) (Immunotech, Beckman Coulter Company, Milan, Italy) was used as the secondary antibody. Slides were then cover-slipped using the Vectashield fluoromount (Vector Laboratorie Inc., Burlingame, CA, USA) and stored at -20°C until analysed for fluorescence.

Fluorimetric determination of the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$

The fluorescent calcium indicator Fura-2 was used to determine the $[Ca^{2+}]_i$ (10) in HFL-1 fibroblasts. Fibroblasts (2.5–$3\text{x}10^4$) grown on 20 mm cover slips were incubated with 10 μM Fura-2 acetoxymethyl ester (Fura-2AM; Fluka, Milan, Italy) and Pluronic F-127 (Invitrogen, Milan, Italy) in medium for 45 min at 37°C. After the addition of known concentrations of BK to the perfusion chamber, the time-course of the cytosolic calcium level was determined as described previously (12). The intracellular free calcium concentration was calculated according to the equation:

$$[Ca^{2+}]_i = bKD \, (R- R\text{min})/(R\text{max}-R)$$

where R is $E340/E380$; Rmin is $E340/E380$ in zero calcium; Rmax is $E340/E380$ in calcium-saturated solution; b is $E380$ in zero calcium/$E380$ in calcium-saturated solution; and $KD = 140$ nM (the dissociation constant of the dye at room temperature) (12). To obtain the parameter values, after each experiment cells were incubated in 10 μM 4-bromo-calcium ionophore A23187 for 20–40 min in a zero-calcium bath (0 calcium plus 1mM EGTA) and then perfused with the saturated Ca^{2+} solution. At the end of this procedure, 5mM $MnCl_2$ was added to the bath to quench the fluorescence of the dye and determine the background values.

Statistical analysis

Statistical evaluation was performed using the statistical software package GraphPad Prism 3.0. The data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of multiple treatment groups was performed using one-way analysis of variance and subsequently Dunnett's two-sided comparison as post-hoc test. Differences between two groups were compared using Student's unpaired t-test. A p-value of <0.05 was considered as statistically significant.

RESULTS

BK-induced α-SMA expression

Endogenous expression of α-SMA in unstimulated fibroblasts shown by Western blot analysis was significantly increased 1.6–2-fold after incubation with BK (1 μM) for 48 h (Fig. 1A and B, 2 and 3). The BK-induced α-SMA expression was completely inhibited (to a level below the constitutive expression) by co-incubation with either BUD (0.1 μM) (p<0.001), FM (0.1 μM) (p<0.001) or the combination of the two drugs (p<0.01) (Fig. 1A and B). In contrast, BUD and FM had no effect on constitutive α-SMA expression in unstimulated fibroblasts (data not shown). When fibroblasts were treated with lower concentrations of the drugs and at a ratio BUD:FM = 10:1 (approximate to that used in Symbicort®), a significant and concentration-dependent reduction of the BK-induced α-SMA expression was observed (Fig. 2).

Immunofluorescence analysis demonstrated that the enhanced α-SMA expression induced by BK was to the result of an increase in the numbers of α-SMA positive (α-SMA$^+$) fibroblasts as well as an increase in polymerization of α-SMA filaments (Fig. 3). In BK-stimulated fibroblasts, BUD and FM, alone and in combination, decreased the numbers of α-SMA$^+$ cells, and a clear reduction in the organization of α-SMA stress fibres was also observed (Fig. 3). In contrast, no such effects of the two drugs were observed in unstimulated fibroblasts (data not shown).

BK-induced B2R expression

The constitutive expression of B2R protein in fibroblasts was up-regulated by 45% after incubation with BK (1 μM) for 48 h (p<0.05) as shown by Western blot analysis (Fig. 4). Co-incubation with BUD (0.1 μM) completely abolished the BK-induced increase in B2R expression (p<0.001), resulting in a level below that of the constitutive expression. In contrast, FM alone (0.1 μM) or in combination with BUD (0.1 μM) had no significant effect on B2R expression (Fig. 4). No effects of the drugs on constitutive B2Rexpression were observed in unstimulated fibroblasts (data not shown).

BK-induced ERK1/2 phosphorylation

Activation of ERK1/2 occurs through phosphorylation of threonine (Thr) 202 and tyrosine (Tyr) 204 by a single upstream MAP kinase (MEK). Exposure of fibroblasts to BK (0.1 μM) for 5 min resulted in a 4-fold increase (p<0.001) of ERK1/2 phosphorylation compared with baseline conditions as shown by Western blot

analysis using a phospho-specific polyclonal antibody recognizing phosphorylated ERK1/2 MAPK (Fig. 5). Pre-incubation for 30 min with BUD (0.1 μM) or FM (0.1 μM) significantly decreased ERK1/2 phosphorylation by 67% (p<0.01) and 49% (p<0.05), respectively (Fig. 5), while pre-incubation for only 15 min with either drug had no effect (data not shown). Pre-incubation for 60 min with BUD did not further increase the inhibition while FM inhibited ERK1/2 phosphorylation completely (p<0.001) back down to baseline level. After 60 min of drug preincubation, almost complete inhibition was also obtained by the combination of the BUD and FM (Fig. 5). The concentration–response study performed after 60 min pre-incubation with the drugs (Fig. 6) showed approximately 60–70% inhibition with all the three BUD concentrations investigated (0.001, 0.01 and 0.1 μM) whereas the effect of FM was concentration-dependent, producing 40% inhibition at 0.001 μM (p<0.05) and complete (100%) inhibition at 0.1 μM (p<0.001).

BK-induced Ca^{2+} mobilization

Fluorimetric analysis demonstrated that in the presence of extracellular Ca^{2+} (CaCl$_2$ 1.8 mM), the addition of BK (1 μM) to the Fura-2-loaded fibroblasts resulted in a rapid increase in [Ca^{2+}]$_i$ that reached a peak (360±16 nM) within 15 seconds and declined to nearly resting level within 10 min (Fig. 7A). The approximate 15-fold elevation of [Ca^{2+}] by BK was significantly reduced by about 40% when fibroblasts were pre-incubated with BUD (0.1 μM) and/or FM (0.1 μM) for 60 min before exposure to BK (p<0.01, for each; Fig. 7B). A significant reduction was also observed when the cells were pre-incubated with either drug for 5, 15 or 30 min (Fig. 7C). Both BUD and FM achieved maximal effects – approximately 70% reduction (p<0.001) – after 30 min pre-incubation. For FM, the time-dependency of the effect was evident; with only 10% and 31% reduction achieved after 5 and 15 min, respectively. For BUD, the time-dependency of the effect was less obvious and the 56% reduction that was achieved after only a 5 min incubation period suggests a rapid, possibly non-genomic mechanism of action of BUD on BK-induced [Ca^{2+}] elevation.

BK-induced p65 NFκB translocation

NFκB activation was evaluated by quantifying p65 NF-κB in the cell nuclei using an immunoblotting assay (in relation to nuclear house-keeping protein lamin A/C). Incubation of fibroblasts with BK (1 μM) for 3 h increased p65 NF-κB protein expression in the nuclear compartment by 63% (p<0.05). This increase was completely prevented when the cells were pre-incubated for 1 h with BUD (0.1 μM) or FM (0.1 μM) or the combination (p<0.05 for all treatments; Fig. 8).

DISCUSSION

This study, performed in HFL-1 fibroblasts, showed that both BUD and FM, either alone or in combination, are capable of totally blocking BK-induced differentiation into myofibroblasts, preventing the BK-induced enhancement of α-SMA expression, the increase in the numbers of α-SMA⁺ cells, as well as the polymerization of α-SMA filaments. BUD also completely inhibited the BK-induced up-regulation of B2R expression while FM alone or in combination with BUD had no effects. These results suggest that other mechanisms than downregulation of B2R expression are involved in blocking BK-induced differentiation of fibroblasts into α-SMA⁺ myofibroblasts by the combination of BUD and FM. The mechanisms most likely to be involved are BK-activated intracellular pathways, such as ERK1/2 phosphorylation, Ca^{2+} mobilization and NFκB translocation; all of which were effectively downregulated by the two drugs. Importantly, the inhibitory effects of BUD and FM demonstrated *in vitro* here were obtained at nanomolar concentrations that are considered therapeutically relevant (22, 23).

The number of myofibroblasts is increased in the airways of asthmatic patients; myofibroblasts are a rich source of tissue matrix proteins and in the airways they play a key role in tissue matrix remodeling leading to increased thickness of the subepithelial basement membrane (24). The demonstration here that the combination of BUD and FM counteracts BK-induced fibroblast differentiation into α-SMA⁺ myofibroblasts *in vitro* is in agreement with the recent findings that inhaled BUD and FM combination therapy significantly attenuated the allergen-induced increase in the number of submucosal tissue myofibroblasts in asthmatic patients (15). However, in that clinical study only the combination therapy had this effect; it was not observed with budesonide monotherapy. Another clinical study has also shown that budesonide and formoterol inhalation therapy in asthmatic patients reversed the increased thickness of the subepithelial basement membrane and the bronchial wall (16) which are the fundamental features of asthma of any severity.

Subepithelial fibrosis in the airways of asthmatic patients may contribute to increased airway contractility. Importantly, α-SMA has been suggested to play a role in the production of contractile force during wound healing and fibrocontractive diseases, exerting tension on cytoskeletal components, such as microtubules and intermediate filaments, organized as a tensegrity structure (12, 25, 26). The present study demonstrated that BUD and FM not only inhibited α-SMA protein expression but also α-SMA organization, a condition that promotes myofibroblast contractility (12). Although we did not evaluate to what extent the two drugs influenced protein translation or post-transcriptional processing of protein synthesis, we showed an effective decrease in the numbers of α-SMA⁺ cells with a clear reduction in α-SMA stress fibre polymerization.

BK exerts most of its effects on fibroblast functions, including cell differentiation, through the activation of B2R, a G protein-coupled receptor (GPCR) constitutively expressed on most cell types and that can be up- or down-regulated by a variety of molecules (2, 27). Physiologically, regulation of the human B2R occurs at the protein level with receptor-mediated ligand internalization following BK binding and being accompanied by a transient loss of surface expression (27, 28). In agreement with a previous report showing that the expression of B2R (and B1R) induced by TNF-α or IL-1β in lung fibroblasts can be inhibited at the post-transcriptional level by dexamethasone (29), we have demonstrated in this study that the BK-induced B2R up-regulation was completely abolished at the protein level by BUD. In contrast, despite the demonstration that long-acting β_2-agonists may down-regulate the TNF-α-mediated induction of cell-surface proteins on lung fibroblasts (30), in the present study we did not observe any significant inhibitory activity of FM alone on BK-induced B2R expression and, on the other hand, we also found a counteracting effect of FM on BUD inhibition of B2R expression. We may suggest that FM, acting through the cAMP-mediated intracellular pathways (31) able to regulate B2R transcriptional genes (32), has the capability to functionally antagonize the molecular mechanisms induced by BUD in down-regulating B2R expression.

The intracellular pathways that regulate the expression of the B2R gene and those which are activated by B2R stimulation in human cells are only partially known (27, 29). In human osteoblastic cell line and gingival fibroblasts, TNF-α-induced BR2 gene expression involves NFκB and p38 MAPK pathways (13). Stimulation of B2R engages a number of intracellular events and pathways, including phosphorylation of MAPK, activation of the transcription factor NFκB and mobilization of $[Ca^{2+}]_i$, all of which are reported to promote fibroblast differentiation and activation (8, 11-13), and have been the focus of attention of this study. Indeed, as also demonstrated in this report, BK induces ERK 1/2 phosphorylation, elevation of $[Ca^{2+}]_i$ levels and NFκB activation in human lung fibroblasts. We have demonstrated here that BUD and FM, either alone or in combination, are able to abrogate BK-induced ERK1/2 phosphorylation and p65 translocation from the cytosol to the nucleus, and to decrease significantly $[Ca^{2+}]_i$ mobilization. When administered in combination, BUD and FM exerted additive effects on NFκB activation and ERK1/2 phosphorylation. This suggests that the inhibitory effects exerted by the two drugs on BK-activated pathways investigated are likely mediated by different mechanisms. This also seems to be valid for the effects on BK-induced increases in $[Ca^{2+}]_i$ mobilization, where the degree of inhibition was dependent on the pre-incubation time with FM but not with BUD. While FM is probably able to reduce $[Ca^{2+}]_i$ mobilization via the cAMP/PKA signalling pathway (33, 34), our finding that BUD exerted a powerful (>50%) inhibition of $[Ca^{2+}]_i$ mobilization after only 5-min pre-incubation, suggests the

involvement of rapid, non-genomic mechanisms, such as the inhibition of calcium-mobilizing cytoplasmic messengers (34, 35).

CONCLUSION

In summary, both BUD and FM, alone and in combination, at therapeutically relevant concentrations, effectively inhibited the BK-induced differentiation of fibroblasts into α-SMA$^+$ myofibroblasts and the intracellular signalling pathways involved in fibroblast activation. Since myofibroblasts are key cells responsible for enhanced matrix production and deposition in the airways of asthmatic patients, these results suggest that BUD and FM therapy has the potential to inhibit fibroblast-dependent airway matrix remodelling in asthma and by that also to counteract the long-term deterioration in lung function described in this disease. Further clinical investigations are warranted to confirm the results of this *in vitro* study.

DECLARATION OF INTEREST

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FIGURE CAPTIONS

FIGURE 1.- Effects of BUD and/or FM on BK-induced expression of α-SMA protein in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μM in the presence or absence of BUD and/or FM (both 0.1 μM), and α-SMA protein expression was then evaluated by immunoblot analysis. **A:** Representative blottings for α-SMA and the house-keeping protein β-actin; arrows indicate the molecular size of α−SMA (43 kDa) and β-actin (42 kDa) characteristic bands. **B:** After densitometric analysis, data were normalised to β-actin and plotted as mean ± SEM. The different experimental conditions are reported on the abscissa whereas the α-SMA:β-actin ratio is shown on the ordinate. ¶¶p<0.01 *vs.* unstimulated cells; **p<0.01 and ***p<0.001 *vs.* cells stimulated with BK in the absence of the drugs.

FIGURE 2.- Effects of BUD and FM in combination on BK-induced expression of α-SMA protein in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μM in the presence or absence of BUD (0.001–0.1 μM) and FM (0.0001–0.01 μM) at BUD:FM dose ratio of 10:1 (approximate to that used in Symbicort®). **A:** Representative blotting for α-SMA and the house-keeping protein β-actin; arrows indicate the molecular size of the α−SMA (43 kDa) and β-actin (42 kDa) characteristic bands. **B:** After densitometric analysis, data were normalised to β-actin and plotted as mean ± SEM. The different experimental conditions are reported on the abscissa whereas the α-SMA:β-actin ratio is shown on the ordinate.

¶p<0.05 *vs.* unstimulated cells; *p<0.05, **p<0.01 and ***p<0.001 *vs.* cells stimulated with BK in the absence of the drugs.

FIGURE 3.- Representative photomicrographs obtained by confocal microscopy after immunofluorescence staining, showing the effect of BUD and/or FM on the BK-induced on α-SMA cytoskeletal organization in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μM (or vehicle) in the presence or absence of BUD and/or FM (both 0.1 μM). Arrows indicate the polymerization of α−SMA⁺ cytoskeletal fibres. Original magnification 20x.

FIGURE 4.- Effects of BUD and/or FM on BK-induced B2R expression in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μM (or vehicle) in the presence or absence of BUD and/or FM (both 0.1 μM), and α-SMA protein expression was then evaluated by immunoblot analysis. **A:** Representative blotting for B2R

and the house-keeping protein β-actin; arrows indicate the molecular size of the B2R (42 kDa) and β-actin (42 kDa) characteristic bands. **B:** After densitometric analysis, data were normalised to β-actin and plotted as mean ± SEM. The different experimental conditions are reported on the abscissa whereas the B2R:β-actin ratio is shown on the ordinate. ¶$p<0.05$, *vs.* unstimulated cells; ***$p<0.001$ *vs.* cells stimulated with BK in the absence of the drugs.

FIGURE 5.- Effects of BUD and FM on ERK1/2 activation in HFL-1 fibroblasts induced by BK. Cells were pre-treated with BUD and/or FM (both 0.1 μM) for 30 or 60 min, and then stimulated for 5 min with BK 0.1 μM. **A:** Representative blotting for phosphorylated (P-) and total (tot-) ERK1/2; arrows indicate the molecular size of the P-ERK1/2 (42-44 kDa) and tot-ERK1/2 (42-44 kDa) characteristic bands. **B:** After densitometric analysis, data were plotted as mean ± SEM. The different experimental conditions are reported on the abscissa whereas the P-ERK1/2 and tot-ERK1/2 ratios on the ordinate.

¶¶¶$p<0.001$ *vs.* unstimulated cells; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ *vs.* cells stimulated with BK in the absence of the drugs.

FIGURE 6.- Effects of BUD and FM on ERK1/2 phosphorylation in HFL-1 fibroblasts induced by BK.

Cells were pre-treated with BUD or FM (both 0.001 to 0.1 μM) for 60 min, and then stimulated for 5 min with BK 0.1 μM. **A:** Representative blotting for phosphorylated- (P-) and total (tot-) ERK1/2; arrows indicate the molecular size of the P-ERK1/2 (42-44 kDa) and tot-ERK1/2 (42-44 kDa) characteristic bands. **B:** After densitometric analysis, data were plotted as mean ± SEM. The different experimental conditions are reported on the abscissa whereas the P-ERK1/2 and tot-ERK1/2 ratios on the ordinate. ¶¶¶$p<0.001$ *vs.* unstimulated cells; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ *vs.* cells stimulated with BK in the absence of the drugs.

FIGURE 7.- Effects of BUD and/or FM on BK-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in HFL-1 fibroblasts. **A:** BK-induced (1 μM) rapid increase in intracellular $[Ca^{2+}]_i$. **B:** Cells were cultured in the presence of BUD and/or FM (both 0.1 μM) for 60 min, and then exposed to BK 1 μM. **C:** Time-dependent effects of BUD and FM; cells were pre-incubated with BUD or FM (both 0.1 μM) for 5, 15 and 30 min before exposure to BK 1 μM, and $[Ca^{2+}]_i$ levels were measured by fluorimetric analysis. The different experimental

conditions are reported on the abscissa and the intracellular calcium concentrations ($[Ca^{2+}]_i$) on the ordinate. Data are presented as means ± SEM.

¶¶¶p<0.001 *vs.* unstimulated cells; **p<0.01 and ***p<0.001 *vs.* cells stimulated with BK in the absence of the drugs.

FIGURE 8.- Effects of BUD and/or FM on p65 NFκB translocation into the nucleus induced by BK in HFL-1 fibroblasts. Cells were pre-treated for 1 h with BUD and/or FM (both 0.1 μM) before stimulation with BK 1 μM for 3 h. **A:** Representative blotting, showing p65 NFκB and the house-keeping protein lamin A/C levels in the nuclear compartment; arrows indicate the molecular size of p65 (60 kDa) and lamin A/C (65-74 kDa) characteristic bands. **B:** After densitometric analysis data were normalized to lamin and shown as anti-p65/anti-lamin ratio. The different experimental conditions are reported on the abscissa and the anti-p65/anti-lamin ratio on the ordinate, and shown as mean ± SEM. ¶p<0.05 *vs.* unstimulated cells; *p<0.05 *vs.* cells stimulated with BK in the absence of the drugs.