

Chapter 4

Interactive Inhibitory Effects of Formoterol and Montelukast on Activated Human Neutrophils.



4.1 INTRODUCTION

Anti-inflammatory chemotherapy, most commonly inhaled corticosteroids, is the cornerstone of the pharmacotherapy of bronchial asthma (McFadden, 1998). In patients not controlled on corticosteroids alone, the guideline recommendation is that additional controller therapy should be added, either a long acting beta(2)-adrenoreceptor agonist (LABA) or a cysteinyl leukotriene receptor (cysLTR) antagonist (Van Weel *et al*, 2008). In patients who are still not adequately controlled, a combination of controller medications is added to the inhaled corticosteroids (Van Weel *et al*, 2008). With the possible exception of effects of corticosteroids on beta(2)- receptor expression and function (Johnson, 2002; Johnson, 2006; Lovén *et al*, 2007), the clinical benefit of this triad of agents is thought to result from the collective effects of their distinct mechanisms of therapeutic activity, as opposed to meaningful, beneficial interactions between them.

Corticosteroids possess broad spectrum anti-inflammatory activity, while LABAs and montelukast function primarily as bronchodilators and through antagonism of cysLTRs respectively (Capra *et al*, 2006; Barnes, 2007). There is, however, evidence that LABAs and montelukast, in addition to their conventional therapeutic activities, possess anti-inflammatory properties (Tintinger *et al*, 2000; Johnson, 2002; Anderson *et al*, 2009; Theron *et al*, 2009). If operative *in vivo*, these may complement the anti-inflammatory actions of corticosteroids in controlling severe

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asthma, especially those types of asthma in which the seemingly corticosteroidresistant neutrophil (Barnes, 2007) is the dominant type of inflammatory cell (MacDowell & Peters, 2007).

The pro-inflammatory activities of neutrophils, as well as other types of immune and inflammatory cells, are suppressed by pharmacological agents which elevate intracellular cyclic AMP (Moore & Willoughby, 1995). Because these cells possess beta(2)-adrenoreceptors, they are amenable to the anti-inflammatory actions of beta(2)-agonists, including the selective, long-acting agent, formoterol (Tintinger *et al*, 2000; Johnson, 2002). As mentioned in the previous chapter, montelukast increases intracellular cyclic AMP by a mechanism distinct from antagonism of cysLTRs, which apparently involves non-specific inhibition of cyclic nucleotide phosphodiesterases [PDEs], resulting in cyclic AMP-dependent inhibition of neutrophil pro-inflammatory activity. Although untested, it is plausible that the combination of formoterol and montelukast may exert augmentative, cyclic AMPdependent anti-inflammatory activity through the respective interactions of these agents with beta(2)-adrenoreceptors and PDEs, especially PDE4 (Wang *et al*, 1999).

In the current study, the effects of formoterol and montelukast, individually and in combination, on several pro-inflammatory activities of activated neutrophils in relation to alterations in cyclic AMP and Ca²⁺ handling by the cells, have been investigated. In a limited series of experiments dexamethasone and salbutamol were included for comparison.



4.2 MATERIALS AND METHODS

4.2.1 Chemicals and reagents

Formoterol fumarate dehydrate and dexamethasone were purchased from Sigma-Aldrich (Pty)Ltd, Johannesburg, South Africa, and montelukast provided by Merck Research Laboratories, Rahway, NJ, USA, while salbutamol and rolipram were provided by GlaxoSmithKline plc, Stockley Park, West London, UK. With the exception of salbutamol (water soluble) all agents were dissolved to a stock concentration of 10 mmol.L⁻¹ in dimethyl sulphoxide (DMSO) and used at final concentrations of 1 and 10 nmol.L⁻¹ (formoterol), 2 μ mol.L⁻¹ (montelukast), 100 nmol.L⁻¹ (salbutamol), 1 μ mol.L⁻¹ (dexamethasone), and 1 μ mol.L⁻¹ (rolipram) in the various assays of neutrophil function described below. The final concentrations of DMSO in each assay ranged from 0.1-0.2% and appropriate solvent controls were included with each experimental system.

The concentrations of montelukast used in the current study (1-2 μ mol.L⁻¹), closely approximate peak serum levels of about 1 μ mol.L⁻¹ attained during chemotherapy with this agent (Knorr *et al*, 2001). The concentration of formoterol in the airways is, however, more difficult to ascertain. Assuming a maximum single dose of 24 μ g and lung deposition of 18.6%, the total amount of formoterol reaching the airways would be approximately 4.5 μ g (Farr *et al*, 1995). Given a tidal volume of 500 mL, this would equate to a local concentration of 9 ng·mL⁻¹, which is equivalent to 11 nmol.L⁻¹, close to the highest concentration of 10 nmol.L⁻¹ used in the current



study. Unless indicated, all other chemicals and reagents were purchased from Sigma-Aldrich.

The chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μ mol.L⁻¹ final), in combination with cytochalasin B (CB, 3 mmol.L⁻¹ final) was used to activate the neutrophils. This relatively strong activator (FMLP/CB) was used because of the anti-inflammatory potency of formoterol and montelukast. An additional chemoattractant, platelet-activating factor (PAF, 200 nmol.L⁻¹) was used in an additional, limited series of experiments.

4.2.2 Preparation of neutrophils

The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all blood donors. These cells were isolated according to the method described in Chapter 2, 2.2.1.

4.2.3 Superoxide generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) procedure. Briefly, neutrophils (10^6 cells) were preincubated without and with montelukast (2 µmol.L⁻¹) in 900 µL Hanks' balanced salt solution (HBSS, pH 7.4, indicator-free; Highveld Biological (Pty) Ltd, Johannesburg, South Africa) containing 0.2 mmol.L⁻¹ lucigenin. Following preincubation, formoterol (1 or 10 µmol.L⁻¹) was added to the cells (or an equal



volume of solvent to control systems) followed 20 sec later by FMLP/CB, and LECL responses recorded using a Lumac Biocounter (model 2010, Lumac Systems Inc, Titusville, FL,USA). The final volume in each vial was 1 mL, and the results, which are expressed in relative light units (rlu), are the peak values for FMLP/CB– activated systems that were reached 40-50 sec after the addition of the activator. There were 4 systems in each experiment: i) neutrophils only (control); ii) neutrophils + formoterol; iii) neutrophils + montelukast; and iv) neutrophils + montelukast + formoterol.

4.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule enzyme, elastase. Neutrophils were incubated at a concentration of 2 x 10^{6} ·mL⁻¹ in HBSS without and with montelukast (2 µmol.L⁻¹) for 10 min at 37°C followed by addition of formoterol (1 or 10 nmol.L⁻¹) or solvent control to the relevant systems and 20 sec later by FMLP/CB. The tubes were incubated and the elastase assays performed according to methods described in in chapter 2, 2.2.5. The effects of the corticosteroid, dexamethasone (1 µmol.L⁻¹), on elastase release by FMLP/CB-activated neutrophils were also investigated in a more limited series of experiments. The corticosteroid was present with the cells during the 10 min preincubation period.



4.2.5 CR3 expression

Expression of CR3 on resting and FMLP/CB-activated neutrophils in the absence or presence of formoterol (10 nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹), individually and in combination, was measured flow cytometrically. Neutrophils (1 x 10⁶·mL⁻¹, final) were incubated in HBSS with and without montelukast for 10 min at 37°C followed by the addition of formoterol or solvent control to the relevant systems and 20 sec later by FMLP/CB. The tubes were incubated for 1 min and the reactions stopped by dilution of the cells in ice-cold medium. Phycoerythrin-labelled monoclonal antibody to CD11b (Beckman Coulter, Miami, FL, USA) was added in a 10 μ L volume to 1 mL of cell suspension and CR3 expression analysed using an Epics Altra Flow Cytometer equipped with a water-cooled Enterprise Laser (Beckman Coulter) and the results expressed as mean fluorescence intensity.

4.2.6 Spectrofluorimetric measurement of cytosolic Ca²⁺

Fura-2/AM was used as the fluorescent, Ca^{2+} -sensitive indicator for these experiments (Grynkiewicz, Poenie & Tsien, 1985). Neutrophils $(1x10^{7} \cdot mL^{-1})$ were incubated with fura-2/AM (2 µmol.L⁻¹) for 30 min at 37°C in PBS, washed and resuspended in HBSS. The fura-2-loaded cells (2 x $10^{6} \cdot mL^{-1}$) were then preincubated for 5 min at 37°C without and with montelukast (2 µmol.L⁻¹) after which they were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500nm respectively. After a stable baseline



was obtained (± 1 min), formoterol (10 nmol.L⁻¹ only) or solvent control was added to the relevant systems followed 20 sec later by FMLP/CB, and alterations in fluorescence intensity monitored over a 5-10min time course. Cytosolic Ca²⁺ concentrations were calculated as described previously (Grynkiewicz, Poenie & Tsien, 1985).

In a more limited series of experiments, the following were also investigated: i) the effects of the short-acting beta(2)-receptor agonist, salbutamol (100 nmol.L⁻¹, final, predetermined in preliminary experiments) without and with montelukast (1 μ mol.L⁻¹) on PAF (200 nmol.L⁻¹)-activated increases in cytosolic Ca²⁺ concentrations in neutrophils; ii) the effects of formoterol (10 nmol.L⁻¹) alone or in combination with the prototype PDE₄ inhibitor, rolipram (fixed, final concentration of 1 μ mol.L⁻¹); and iii) the effects of dexamethasone (1 μ mol.L⁻¹) on FMLP/CB-activated Ca²⁺ fluxes in neutrophils. Montelukast, rolipram and dexamethasone were present with the cells during the 10 min preincubation period, while salbutamol or formoterol were added 20 sec prior to the activation of the cells.

4.2.7 Measurement of leukotriene B₄ (LTB₄) and cyclic AMP (cAMP)

Competitive binding immunoassay procedures (Correlate-EIATM, Assay Designs Inc., Ann Arbor, MI, USA) were used to measure LTB₄ in the supernatants of FMLP/CB-activated neutrophils, while cAMP was assayed in the extracts of unstimulated cells in the absence and presence of formoterol and montelukast individually and in combination. In the case of LTB₄, neutrophils (2 x 10^{6} ·mL⁻¹)



were preincubated for 10 min at 37°C in the absence and presence of montelukast (2 μ mol.L⁻¹) after which formoterol (1 or 10 nmol.L⁻¹) was added to the relevant systems, followed 20 sec later by FMLP/CB. Incubation was terminated 3 min later by the addition of an equal volume of ice-cold HBSS to the tubes which were then held in an ice bath prior to pelletting the cells by centrifugation. The cell-free supernatants were then diluted (1:8) and assayed for LTB₄, with the results expressed as pg LTB₄ ·10⁷ cells⁻¹. As with the other assays of neutrophil pro-inflammatory activity, each experiment consisted of 4 systems: i) neutrophils only (control); ii) neutrophils + formoterol; iii) neutrophils + montelukast; and iv) neutrophils + formoterol + montelukast. In a more limited series of experiments, the following were also investigated: i) the effects of salbutamol (100 nmol.L⁻¹) without and with montelukast (1 μ M) on the production of LTB₄ by PAF (200 nmol.L⁻¹)-activated neutrophils; and ii) the effects of dexamethasone (1 μ mol.L⁻¹)

In the case of cAMP, neutrophils (2 x $10^{6} \cdot mL^{-1}$) were preincubated for 10 min at 37°C without or with montelukast (2 µmol.L⁻¹), after which formoterol (10 nmol.L⁻¹ only) was added to the relevant systems and the reactions terminated 20 sec later by the addition of an equal volume of ice-cold HBSS to the tubes. Following centrifugation, the supernatants were discarded and cAMP extracted from the cell pellets by addition of 0.1M HCl for 15 min followed by centrifugation to remove the cell debris and the supernatants decanted and assayed for cAMP. These results are expressed as pmol cAMP·10⁷ cells⁻¹.



4.2.8 Statistical analysis

With the exception of the results of the fura-2 fluorescence experiments, some of which are presented as representative traces, the results of each series of experiments are presented as the mean values \pm SEM, where n = the number of different donors used in each series of experiments, with the number of replicates for each drug concentration and drug-free control system for each experiment shown in the figure legends. Levels of statistical significance were determined by comparing the absolute values for each drug-treated system with the corresponding values for the relevant drug-free control systems for each assay using repeated measures ANOVA with an appropriate multiple comparisons posttest.

4.3 RESULTS

4.3.1 Superoxide production, elastase release, and leukotriene B₄

These results for neutrophils activated with FMLP/CB in the absence and presence of formoterol (1 and 10 nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹) individually and in combination are shown in Figure 4.1 (page 127). Treatment of neutrophils with formoterol or montelukast significantly (*P*<0.05) attenuated the production of superoxide and LTB₄ in particular, as well as the release of elastase, with the combination, especially in the case of 1 nmol.L⁻¹ formoterol, being significantly (*P*<0.05) more effective in most cases, albeit additive rather than synergistic, than either of the individual agents. The exception was superoxide production for which



the effects of the combination differed only modestly from those of montelukast alone. The magnitude of inhibition of elastase release observed in the presence of montelukast, although statistically significant, was less than that reported in Chapter 3, probably because of the higher concentration of cytochalasin B used in the current study.

Treatment of neutrophils with dexamethasone had minimal effects on the production of LTB₄ and release of elastase from FMLP/CB-activated neutrophils. In the case of elastase the values for cells activated with FMLP/CB in the absence and presence of dexamethasone were 950 ± 15, and 932 ± 15 munits elastase $\cdot 10^7$ cells⁻¹ respectively. The corresponding values for LTB₄ release were 7213 ± 110 and 7242 ± 150 pg LTB₄ $\cdot 10^7$ cells⁻¹ (data from 3 separate experiments). The effects of salbutamol (100 nmol.L⁻¹) and montelukast (2 µmol.L⁻¹) individually and in combination on the release of LTB₄ by PAF-activated neutrophils were also investigated. The results for unstimulated cells and for PAF-activated control cells and cells treated with salbutamol alone, montelukast alone, or the combination of these agents were 24 ± 2, 681 ± 29, 335 ± 55, 132 ± 38, and 65 ± 17pg LTB₄ $\cdot 10^7$ cells⁻¹ respectively (*P*< 0.05 for comparison of each drug-treated system with the control system and for the combination with the individual agents).

4.3.2 CR3 expression

The level of CR3 expression for resting neutrophils was 27 ± 2 mean fluorescence intensity. The corresponding levels of expression for control, FMLP/CB-activated



cells and for the corresponding systems treated with formoterol (10 nmol.L⁻¹) only, montelukast (2 μ mol.L⁻¹) only, or the combination of these two agents were 92 ± 2, 71 ± 3, 81 ± 2 and 60 ± 3 mean fluorescence intensity respectively. The levels of CR3 expression were significantly less (*P*<0.05) than those of the control system for all 3 drug-treated systems, while the levels of CR3 expression in the system treated with both formoterol and montelukast was significantly less (*P*<0.05) than that of the systems treated with the individual drugs.

4.3.3 Cyclic AMP

These results are shown in Figure 4.2 (page 128). Treatment of neutrophils with either formoterol (10 nmol.L⁻¹) for 20 sec, or montelukast (2 μ mol.L⁻¹) resulted in significant (*P* <0.05) elevations in intracellular cAMP. Although higher than that observed with the individual agents, the increase in cAMP which resulted from treatment of the cells with the combination of montelukast and formoterol was not significantly different from that observed with the individual agents (Figure 4.2, page 128).

4.3.4 Cytosolic calcium

The results shown in Figure 4.3 (page 129) are traces from a representative experiment which depicts the alterations in cytosolic Ca^{2+} (fura-2 fluorescence) following activation of the cells with FMLP/CB in the absence and presence of formoterol (10 nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹) individually and in combination. Activation of the cells with FMLP/CB resulted in an abrupt increase in



cytosolic Ca²⁺, with the peak response being sustained for 1-2 min, followed by a gradual subsidence which levelled off within 1-2 min reaching a sustained plateau at a level considerably higher than the pre-activation, basal value for cytosolic Ca²⁺. Treatment of the cells with either montelukast (2 μ mol.L⁻¹) for 5 min or formoterol (10 nmol.L⁻¹) for 20 sec prior to the addition of FMLP/CB caused partial, but significant (*P*<0.05) attenuation of the peak, plateau cytosolic Ca²⁺ response, while accelerating the rate of decline, and decreasing the magnitude of the second, sustained plateau response. Again, the combination of montelukast and formoterol was found to be significantly (*P*<0.05) more effective than the individual agents in promoting clearance of Ca²⁺ from the cytosol of FMLP/CB-activated neutrophils. These results are shown in Table 4.1 (page 125). Similar results (not shown) were obtained with the non-selective PDE inhibitor, rolipram (1 µmol.L⁻¹), in the presence or absence of formoterol (10 nmol.L⁻¹).

Addition of PAF (200 nmol.L⁻¹) to neutrophils resulted in an abrupt increase in cytosolic Ca²⁺, which was followed by a sustained plateau phase and subsequent gradual decline towards basal levels. Pretreatment of neutrophils with salbutamol (100 nmol.L⁻¹) 20 sec prior to addition of the stimulant did not alter the magnitude of the initial peak response, or the duration of the sustained plateau phase. However, the rate of decline in cytosolic Ca²⁺ concentrations was accelerated. In the presence of montelukast (1 μ mol.L⁻¹), the initial plateau phase was markedly attenuated and the rate of decline in cytosolic Ca²⁺ concentrations increased significantly. The combination of salbutamol and montelukast was more effective



than either agent alone. These results are shown in Figure 4.4 (page 130) and Table 4.2 (page 126). Dexamethasone did not detectably alter Ca²⁺ fluxes in FMLP/CB-activated neutrophils (results not shown).

4.4 **DISCUSSION**

Add-on therapy with montelukast has been reported to confer benefit on patients whose asthma is poorly controlled with inhaled corticosteroid monotherapy, or with the combination of a LABA and an inhaled steroid (Dempsey *et al*, 2000; Currie *et al*, 2003; Dupont *et al*, 2005; Dal Negro *et al*, 2009; Keith *et al*, 2009; Korn *et al*, 2009). In this setting, it is believed that montelukast targets inflammatory mechanisms, specifically those orchestrated by cysteinyl leukotrienes and their receptors, thereby complementing the therapeutic actions of inhaled corticosteroids and beta(2)-agonists. However, this may not be the only mechanism by which add-on therapy with montelukast confers benefits on patients with poorly controlled asthma. The effects of montelukast on PDEs (described in the previous chapter) raise the possibility that this agent may act in concert with the other anti-asthma agents, particularly beta(2)-agonists, to augment cAMP-mediated anti-inflammatory activity, and possibly bronchodilatory activity.

To test this hypothesis, we investigated the effects of formoterol (1 nmol.L⁻¹ and 10 nmol.L⁻¹) and montelukast (1-2 μ mol.L⁻¹), individually and in combination on the pro-inflammatory activities of human neutrophils. Treatment of neutrophils with

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either agent resulted in significant inhibition of the generation of superoxide in particular, as well as expression of CR3 and release of elastase following activation of the cells with FMLP/CB, with the combination being more effective than the individual agents, most notably in the case of CR3 expression and elastase release, the effects being additive as opposed to synergistic. In asthma, phagocyte-derived reactive oxygen species and elastase have been identified as possible mediators of bronchial hyperactivity and obstruction, and airway remodelling respectively (Lee *et al*, 2006; Mak & Chan-Yeung, 2006), while interference with CR3 expression will restrict the migration of neutrophils into the airways.

In the case of production of LTB₄ by activated neutrophils, the individual agents were found to be extremely potent inhibitors of production of this eicosanoid, with small, but nevertheless significant augmentative effects observed with the combination of formoterol and montelukast. Notwithstanding the well-recognised involvement of cysteinyl leukotrienes in asthma, it is noteworthy that LTB₄ also appears to play an important role in the pathogenesis of severe persistent asthma, as well as aspirin- and exercise-induced asthma, allergic rhinitis and atopic dermatitis (Ohnishi, Miyahara & Gelfand, 2008). Moreover, corticosteroids, which generally do not affect LTB₄ production by immune and inflammatory cells, have been reported to upregulate the expression of the BLT1 receptor on corticosteroid-resistant cells such as neutrophils, monocytes and effector memory CD8⁺ T cells (Obinata *et al*, 2003; Lee *et al*, 2006; Ohnishi *et al*, 2008; Ohnishi, Miyahara & Gelfand, 2008). In the current study, dexamethasone, an agent which does not



elevate intracellular cAMP concentrations, had no detectable effects on either elastase or LTB₄ release by activated neutrophils.

Although of limited relevance in the treatment of chronic asthma, or asthma in which inflammation is well-controlled, the effects of the short-acting beta(2)-agonist, salbutamol, on LTB₄ production by neutrophils activated with PAF, a less potent stimulus than FMLP/CB, were also measured. Salbutamol also inhibited the production of LTB₄ by PAF-activated neutrophils which was augmented by inclusion of montelukast, probably by interactive cAMP-dependent mechanisms as described in the previous chapter.

To probe the mechanism of formoterol/montelukast-mediated inhibition of the proinflammatory activity of neutrophils, the effects of these agents on alterations in cAMP and cytosolic Ca²⁺ were investigated. Exposure of neutrophils to either formoterol or montelukast was accompanied by substantial increases in cAMP. In the case of montelukast, these were somewhat higher than reported in the previous chapter. This is most likely attributable to the longer exposure of the cells to the drug (10 min in the current study compared to 5 min in the previous study). It is possible that longer exposure times may have revealed significant effects at lower concentrations of montelukast; however, these experiments were not performed. Although the magnitude of the increase in cAMP observed with the combination was slightly higher than that observed with the individual agents, the difference did not achieve statistical significance. This may be due to the fact that a relatively brief exposure time (20 sec) was used at which the effects of formoterol



were maximal; longer exposure times may have revealed significant effects of the combination.

In neutrophils, the anti-inflammatory actions of agents which elevate intracellular cAMP are achieved primarily by activation of cAMP-dependent protein kinase (PKA). PKA in turn accelerates restoration of Ca^{2+} homeostasis and down-regulation of pro-inflammatory activities following exposure of the cells to receptor-linked, Ca^{2+} mobilizing stimuli by several mechanisms including: phosphorylative inactivation of phospholipase C (Ali *et al*, 1998); inactivation of inositol triphosphate (IP₃) receptors on intracellular Ca^{2+} stores (Bai & Sanderson, 2006); up-regulation of the Ca^{2+} sequestering/resequestering endo-membrane Ca^{2+} -ATPase (Anderson *et al*, 2000); and inhibition of p38 MAP kinase, resulting in interference with the activation of 5-lipoxygenase (Flamand *et al*, 2002) and attenuation of an autocrine, LTB₄-mediated secondary wave of Ca^{2+} uptake by the cells (Steel *et al*, 2007).

In the current study, formoterol, as reported previously (Tintinger *et al*, 2000), and montelukast, facilitated the clearance of Ca^{2+} from the cytosol of neutrophils activated with FMLP/CB without affecting mobilization of the cation from intracellular stores. The two agents were most effective when used in combination. These observations are compatible with a mechanism whereby formoterol and montelukast, by targeting beta(2)-adrenoreceptors and PDEs respectively, exert their individual and combined anti-inflammatory activities via cAMP-dependent, accelerated restoration of Ca^{2+} homeostasis. This contention is supported by the following observations: i) exposure of FMLP/CB-activated neutrophils to the type 4



phosphodiesterase inhibitor, rolipram in combination with formoterol resulted in decreases in cytosolic Ca^{2+} concentrations similar to those observed with montelukast and formoterol; and ii) montelukast potentiated the effects of salbutamol on clearance of Ca^{2+} from the cytosol of PAF-activated neutrophils. In keeping with its lack of effects on release of elastase and LTB₄, dexamethasone did not affect Ca^{2+} fluxes in fMLP/CB-activated neutrophils.

In conclusion, the results of the current study identify an interactive, cAMPdependent mechanism by which formoterol and montelukast may support the antiinflammatory actions of inhaled corticosteroids by targeting the intransigient neutrophil in patients with poorly controlled asthma.



Table 4.1: Peak cytosolic Ca^{2+} concentrations, duration of the plateau phase of the peak cytosolic Ca^{2+} response, and area under the curve measured in neutrophils activated with FMLP/CB in the absence and presence of formoterol (10 nmol.L⁻¹) and montelukast (2 µmol.L⁻¹) individually and in combination.

System	Peak cytosolic Ca ²⁺ concentration (nM)	Duration of peak plateau phase (min)	Area under the curve (cm ²) ⁺
FMLP/CB only (Control)	420 ± 11	1.3 ± 0.1	7.6 ± 0.34
FMLP/CB + formoterol	435 ± 7	0.9 ± 0.1*	$6.3 \pm 0.4^*$
FMLP/CB + montelukast	420 ± 15	0.9 ± 0.1*	$6.7 \pm 0.4^{*}$
FMLP + formoterol + montelukast	415 ± 13	0.4 ± 0.1**	5.4 ± 0.4**

The results of 4 separate experiments using cells from 4 different donors are presented as the mean values \pm SEM.

- ⁺ Measured 2 min after the addition of FMLP/CB
- * *P*<0.05 for comparison with the control system
- ** *P*<0.05 for comparison of the combination with the individual agents



Table 4.2: Peak cytosolic Ca²⁺ concentrations, duration of the plateau phase of the peak cytosolic Ca²⁺ response, and the magnitude of decrement in cytosolic Ca²⁺ concentrations in neutrophils activated with PAF (200 nmol.L⁻¹) in the presence or absence of salbutamol (100 μ mol.L⁻¹) and montelukast (1 μ mol.L⁻¹) individually and in combination.

System	Peak cytosolic Ca ²⁺ concentration (nM)	Duration of peak plateau phase (min)
PAF only (Control)	455 ± 12	1.4 ± 0.4
PAF + salbutamol	440 ± 14	1.2 ± 0.4
PAF + montelukast	416 ± 22	0.17 ± 0.02*
PAF + salbutamol + montelukast	416 ± 16	0.14 ± 0.02**

The results of 5-16 separate experiments using cells from 4-6 different donors are presented as the mean values \pm SEM.

*P<0.05 for comparison with the control system,

**P<0.05 for comparison of the combination with the individual agents.





Figure 4.1: Effects of formoterol (1 and 10nmol.L⁻¹) and montelukast (2µmol.L⁻¹) individually and in combination on a) the generation of superoxide, b) the release of elastase, and c) production of leukotriene B4 following activation of neutrophils with FMLP in combination with cytochalasin B. The results are expressed as a % of control of a minimum of four different experiments using cells from four different donors. +P<0.05 for comparison of formoterol and montelukast combination with the system treated with montelukast alone; *P<0.05 for comparison of the formoterol and montelukast combination with the system treated with formoterol alone. The absolute values for the responses of control (drug-free), unstimulated neutrophils for superoxide generation, release of elastase and production of LTB4 were 3.782 ± 475 rlu, 34 ± 2 milli-units enzyme per 10^7 cells, and 37 ± 5 pg LTB₄ per 10^7 cells, respectively; the corresponding values for the control systems activated with FMLP/CB were 54.954 ± 1.9 rlu, 980 ± 16 milli-units enzyme per 10^7 cells and 7.926 ± 1.17 pg LTB₄ per 10^7 cells.





Figure 4.2: Effects of formoterol (10nmol.L⁻¹) and montelukast (2µmol.L⁻¹) individually and in combination on neutrophil intracellular cyclic AMP (cAMP) levels. The results are expressed as the mean \pm SEM values (data from 5 different experiments using cells from five different donors). Exposure of the cells to either formoterol or montelukast was accompanied by significant (p<0.05) elevations in cAMP, while the effects of the combination of agents, although greater, did not differ significantly from those of the individual agents. *P<0.05.





Figure 4.3: N-Formyl-L-methionyl-L-leucyl-phenylalanine (FMLP).cytochalasin B (CB)activated fura-2 fluorescence responses of control neutrophils and cells treated with formoterol (10nmol.L⁻¹) and montelukast (2 μ mol.L⁻¹) individually and in combination. FMLP/CB was added as indicated by arrows when a stable baseline was obtained (± 1 min). The traces are from an individual representative experiment (four in a series using cells from four different donors).





Figure 4.4: Effects of salbutamol (100nmol.L⁻¹; _ _) and montelukast (1 μ mol.L⁻¹;.....) alone and in combination (**x x x**) on platelet factor-activated (arrow) neutrophils. ____; controls.