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Phosphodiesterase 4 inhibition of β_2-integrin adhesion caused by leukotriene B_4 and TNF-α in human neutrophils

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ABSTRACT: Phosphodiesterase (PDE)4 inhibition attenuates neutrophilic inflammation in chronic obstructive pulmonary disease. The objective of the present study was to examine the efficacy and mechanism by which PDE4 inhibition blocks adhesion of β_2-integrin to an endothelial counterligand.

Neutrophils (polymorphonuclear leukocytes (PMNs)) were isolated from humans receiving no medication. Adhesion was analysed by myeloperoxidase activity. The effects of cilomilast ± salmeterol on the following were determined: 1) surface CD11b expression; 2) adhesion; 3) intracellular cyclic adenosine monophosphate (cAMP) concentration; and 4) extracellular signal-regulated kinase (ERK)-1/2-mediated group IVA-phospholipase A_2 (gIVA-PLA_2) phosphorylation caused by leukotriene (LT)B_4 or tumour necrosis factor (TNF)-α activation.

Either cilomilast or rolipram ± salmeterol caused concentration-related blockade of LTB_4-induced adhesion to counterligand, but had no effect on TNF-α-activated PMNs. A comparable increase in intracellular cAMP concentration for PMNs activated with LTB_4 and TNF-α was caused by 1 μM cilomilast and 0.1 μM salmeterol. Upregulation of surface CD11b expression and ERK-1/2 phosphorylation were blocked by cilomilast or rolipram ± salmeterol for PMNs activated by LTB_4, but not for cells stimulated by TNF-α. Cilomilast ± salmeterol also blocked gIVA-PLA_2 phosphorylation caused by LTB_4 but not TNF-α.

In conclusion, the current study demonstrates that both leukotriene B_4 and tumour necrosis factor-α upregulate cyclic adenosine monophosphate. However, cyclic adenosine monophosphate does not block β_2-integrin adhesion caused by tumour necrosis factor-α. It was concluded that tumour necrosis factor-α prevents inhibition of extracellular signal-regulated kinase-1/2-mediated group IVA-phospholipase A_2 activation, which is essential for β_2-integrin adhesion in polymorphonuclear leukocytes.

KEYWORDS: Adhesion, inflammation, neutrophils, phosphodiesterase 4

Cyclic nucleotide phosphodiesterases (PDEs) can be divided into 11 families of PDE enzymes (PDE1–PDE11), eight of which generate over 30 different isoforms that are able to metabolise and deactivate the naturally occurring second messenger nucleotide, 3′,5′-cyclic adenosine monophosphate (cAMP) [1–3]. Among the isoforms, PDE4 enzymes selectively hydrolyse cAMP and have a low affinity for cyclic guanosine monophosphate [1–3]. Due to the apparent number of PDE isoenzymes, it has been anticipated that many cell types express more than a single PDE and that the content of these enzymes varies between different cells and tissues [4]. Recently, four genotypes for PDE4 have been identified (PDE4A–PDE4D); PDE4A, PDE4B and PDE4D are particularly abundant in several inflammatory cells, including neutrophils (polymorphonuclear leukocytes (PMNs)) [5, 6], eosinophils [5, 7], macrophages [8, 9] and monocytes [10, 11]. However, the role of PDE4 in regulating cellular functions has not been fully established.

Guinea pig peritoneal eosinophils predominantly express a membrane-bound PDE4, whereas human eosinophils express PDE4 and PDE7 as intracellular isoenzymes [12]. PDE isoenzymes in human PMNs have been incompletely characterised; however, PDE4 appears to be exclusively intracellular, and PDE4 appears to be the sole PDE isozyme that is expressed in its active state in human PMNs [12].

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The inhibitory effects of PDE4 on downregulation of neutrophil and eosinophil functions, *i.e.* cellular adhesion, granule secretion, CD11b expression, and shedding of L-selectin, have been reported previously [5, 13]. PDE4A is localised within cytoplasmic granules of granulocytes and is predominantly translocated to the plasmalemma in response to formyl-Met-Leu-Phe (FMLP) activation; some PDE4 is exocytosed in the activated state [5]. *In vivo* studies have demonstrated the inhibitory effect of rolipram on endothelial transmigration of neutrophils and eosinophils [14, 15]. Other studies have shown that inhibition of PDE4 causes relaxation of hyperreactive airway smooth muscle and blockade of mediator release by inflammatory cells [15, 16].

The present authors have recently demonstrated that rolipram, a PDE4 inhibitor, blocks the synthesis of cysteinyl leukotriene (LT)C_4 by ~50% in eosinophils activated by FMLP [17]. Co-incubation with salmeterol caused synergistic inhibition of this response [17]. The present authors have also demonstrated that rolipram blocks β_2-integrin-mediated adhesion caused by eotaxin in human eosinophils, but not interleukin-5-mediated adhesion *in vitro* [18]. Addition of salmeterol caused additive blockade of adhesion mediated by eotaxin, a G-protein-activating chemokine, in eosinophils [18]. Other investigations have demonstrated that inhibition of PDE4 attenuates airway hyperresponsiveness and airway inflammation in sensitised mice [19, 20]. Several studies have demonstrated attenuation of airway inflammation and airway reactivity by interaction of salmeterol with corticosteroids and steroid-sparing effects of theophylline [21, 22]; however, little is known about the mechanism of action of PDE4 in β_2-integrin-mediated adhesion in human PMNs.

In the current study, the comparative inhibitory effect of PDE4 inhibition is investigated using cilomilast alone and cilomilast in combination with salmeterol on β_2-integrin-mediated adhesion caused by tumour necrosis factor (TNF)-α or LTB_4 in human PMNs *in vitro*.

METHODS

Isolation of eosinophils and PMNs

Eosinophils were isolated from 18 human subjects (aged 20–45 yrs), as previously described [23]. Informed written consent was obtained from all volunteers. None of the subjects had received any medication for $\geq$4 weeks before the study.

Eosinophils were isolated by immunomagnetic depletion of PMNs using a human eosinophil enrichment kit (StemCell Tech, Vancouver, BC, Canada). Purity was $\geq$98%, as assessed by Wright–Giemsa staining.

PMNs were isolated by Ficoll sedimentation, and centrifugation through lymphocyte separation medium (density 1.077 ± 0.002 g·mL^{-1}; Amersham, Arlington Heights, IL, USA). The cell pellet containing PMNs was resuspended in Hank's balanced salt solution (HBSS) buffer+Ca^{2+}/0.2% bovine serum albumin (BSA), and was >99% viable, as assessed by trypan blue dye exclusion.

β_2-Integrin-dependent adhesion of PMNs to ICAM-1 and BSA-coated microplate wells

Measurement of PMN adhesion: verification of BSA surrogate

Microplate wells were coated with 50 μL of either soluble intercellular adhesion molecule (ICAM)-1 or BSA dissolved in coating buffer and incubated overnight at 4°C and washed with buffer prior to use [24]. Adhesion was assessed as residual myeloperoxidase (MPO) activity of adherent cells. PMNs (4×10^4/100 μL HBSS/0.1% gelatine) were added to coated microplate wells and allowed to settle on ice for 10 min. Cells were activated with 10^{-6} M FMLP at 37°C. After 15 min, microplate wells were gently washed with HBSS, and 100 μL of HBSS/0.1% gelatine was added to the wells. MPO substrate (100 μL; 0.01% H_2O_2, 0.167 mg·mL^{-1} *O*-dianizidine dihydrochloride, and 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 5.5) was added to each well. After 30 min, the reaction was terminated by adding 50 μL of 2 M H_2SO_4. A standard curve was generated for each assay using serial dilutions of the original cell suspension. Absorbance was measured at 405 nm (Thermomax; Molecular Devices, Menlo Park, CA, USA). No MPO activity was detected in the cell-free reaction supernatants (ICAM-1 or BSA), confirming that MPO was not present because of spontaneous neutrophil degranulation. Previous investigations have established the time and concentration of FMLP used in this study are optimal for activating granulocyte adhesion [24].

Time-course and concentration–response curves of the effects of cilomilast and/or salmeterol on β_2-integrin-mediated adhesion caused by 10^{-7} M LTB_4 or 30 ng·mL^{-1} TNF-α were generated for subsequent studies (see below). These concentrations of LTB_4 and TNF-α were those approximating maximal β_2-integrin adhesion (see Results).

Blockade of stimulated β_2-integrin-mediated adhesion

The effect of mouse blocking monoclonal antibodies (mAbs) directed against α-CD11a, α-CD11b, α-CD18, and α-CD49d on upregulated β_2-integrin-mediated adhesion caused by FMLP activation was examined. BSA was used as a surrogate protein for ICAM-1 for all subsequent experiments because a similar pattern of adhesion and blockade of adhesion had been validated in this study.

Determination of PDE4 activity

PDE4 activity in quiescent PMNs and eosinophils was analysed by a modified method of THOMPSON and APPLEMAN [25] and WRIGHT *et al*. [26]. Cells (2×10^6 cells) were treated with either buffer alone or 10^{-6} M cilomilast alone (30 min). The enzyme-containing fractions were assayed in a final volume of 200 μL containing 0.5 μM cAMP (28,000 cpm [^{3}H]cAMP) and incubated for 30 min at 37°C. The reaction mixture was terminated by addition of 50 μL of 0.2 M HCl. Further hydrolysis of 5′-[^{3}H]AMP to [^{3}H]adenosine, was facilitate by addition of *Crotalus atrox* snake venom. The eluate from Sephadex A-25 columns was added to a scintillation cocktail, and radioactivity was counted using a Beckman liquid scintillation counter. PDE4 activity was expressed as femtomoles per minute per million cells (fmol·min^{-1}·10^6 cells^{-1}).

Flow cytometic analysis of cell-surface expression of β_2-integrin adhesion

PMNs were pre-incubated with 10^{-10}–10^{-6} M cilomilast (30 min) and/or 10^{-7} M salmeterol (5 min) prior to activation with 10^{-7} M LTB_4 (15 min) or 30 ng·mL^{-1} TNF-α (30 min) and addition of 300 μL of cold fluorescence-activated cell-sorter (FACS) buffer (PBS with 1% BSA and 0.1% NaN_3 solution). The cell pellet was

washed once with FACS buffer prior to incubation with 4 μL CD11b mAb (Endogen, Woburn, MA, USA), or isotype-matched control antibody (Ab) at 4°C for 60 min. After washing twice with FACS buffer, cells were incubated with an excess of fluorescein-5-isothiocyanate-conjugated goat anti-mouse immunoglobulin (Ig) for 30 min at 4°C. Thereafter, 300 μL of 1% paraformaldehyde was added to the cell pellet and kept at 4°C until analysis. Flow cytometry was performed by FACScan (BD Biosciences, Mountain View, CA, USA) and mean fluorescence intensity was determined on ⩾5,000 cells from each sample.

Effect of PDE4- and β_2-adrenoceptor inhibition on PMN adhesion to ICAM-1 surrogate protein

To exclude possible off-target action of cilomilast, PMNs were pre-incubated with either 10^{-10} M to 10^{-6} M cilomilast or rolipram for 30 min and/or 10^{-7} M salmeterol for 5 min prior to activation with 10^{-7} M LTB_4, 30 ng·mL^{-1} TNF-α, or buffer control at 37°C. Adhesion assay was performed as above.

Determination of intracellular cAMP concentration

cAMP was assay by electroimmunoassay (Assay Design, Ann Arbor, MI, USA). PMNs (2.5×10^5 cells per intervention) were pre-incubated with 10^{-10}–10^{-6} M cilomilast and/or 10^{-7} M salmeterol prior to activation with 10^{-7} M LTB_4, 30 ng·mL^{-1} TNF-α, or buffer control at 37°C. Each cell pellet was treated with 0.1 M HCl for cell lysis, and 200 μL para-nitrophenyl phosphate was added as substrate. The cAMP concentration was read on a microplate reader at 405 nm and expressed as picomoles per 2.5×10^5 cells (pmol·2.5×10^5 cells^{-1}).

Immunoblotting analysis: phosphorylation of ERK-1/2, gIVA-PLA_2 and Raf-1

PMNs were pre-incubated with either 10^{-10}–10^{-6} M cilomilast or rolipram and/or 10^{-7} M salmeterol prior to activation with 10^{-7} M LTB_4, 30 ng·mL^{-1} TNF-α, or buffer control at 37°C, and the cell pellet was lysed in 70 μL of disruption buffer. Supernatant (65 μL) was mixed with 14 μL ×6 of sample buffer and boiled for 5 min. Samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis using 10% acrylamide gels under reducing conditions and electrotransfer of proteins was achieved using a semi-dry system. The membrane was blocked with 1% BSA in Tris-buffered saline plus 0.1% Tween-20 (TBS-T) buffer for 60 min prior to addition of 2 μg·mL^{-1} anti-phosphorylated extracellular signal-regulated kinase (ERK)-1/2 Ab (Promega, Madison, WI, USA) or 2 μg·mL^{-1} anti-phosphorylated group IVA-phospholipase A_2 (gIVA-PLA_2) Ab (Ser505; Cell Signaling Technology, Beverly, MA, USA) or 2 μg·mL^{-1} anti-phospho-Raf Ab (Ser259; Cell Signaling Technology). After washing with TBS-T, the membranes were incubated with 1:3,000 dilution of goat anti-rabbit Ig conjugated with horseradish peroxidase and analysed by an enhanced chemiluminescence system (Amersham).

Statistical analysis

Data are expressed as mean±SEM in each group. Student's t-test was used for comparison between two paired groups. Where multiple comparisons were made, differences in concentration–response curves for the same agonist or inhibitor were compared after Bonferroni correction. Variation among more than two groups was tested using ANOVA followed by Fisher's least-protected difference test. A p-value <0.05 was considered to be statistically significant.

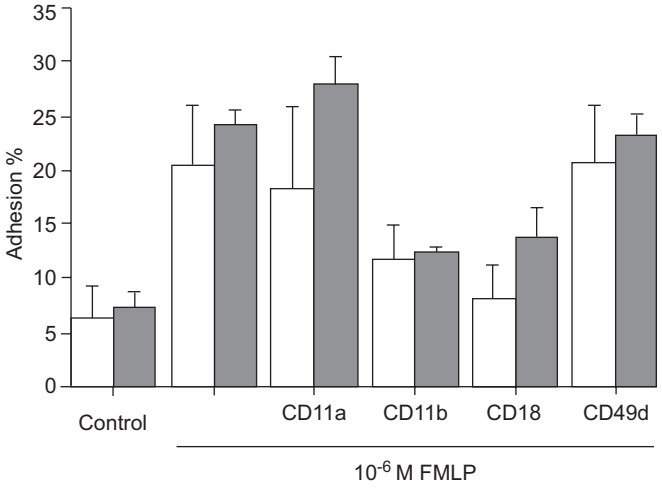

FIGURE 1. Effect of monoclonal antibody (mAb) directed against β_1/β_2-integrins on formyl-Met-Leu-Phe (FMLP)-stimulated neutrophil adhesion to bovine serum albumin (BSA)- (■) or intercellular adhesion molecule (ICAM)-1-coated (□) microplate wells. Polymorphonucleates were pre-incubated with optimal concentration of mAb against β_1- or β_2-integrins or isotype control, and activated with 10^{-6} M FMLP for 15 min in BSA- or ICAM-1-coated wells. Neutrophil adhesion was measured as residual myeloperoxidase activity as described in the Methods section. Each point represents the mean±SEM.

RESULTS

Equivalency of binding of ICAM-1 and BSA surrogate

PMN binding to ICAM-1- or BSA-coated microplate wells

To confirm the equivalency of this method for measuring adhesion of PMNs to ICAM-1, the adhesion of PMNs to BSA as a surrogate protein for the Ig supergene was compared. The number of PMNs adhering to ICAM-1- or BSA-coated wells

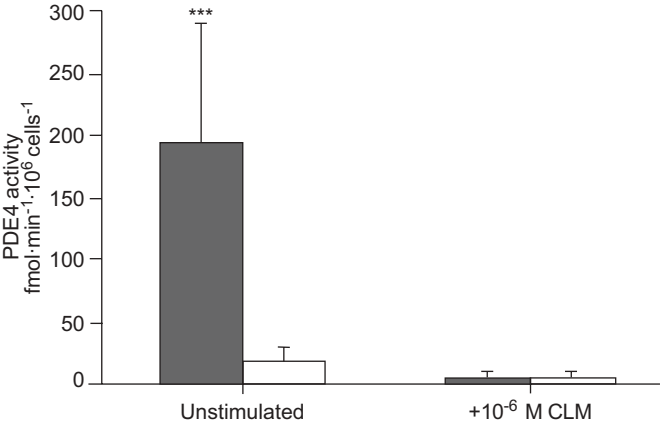

FIGURE 2. Quantitation of phosphodiesterase (PDE)4 activity. Isolated human polymorphonuclear leukocytes (PMN; ■) or eosinophils (EOS; □) were treated with buffer alone or with 10^{-6} M cilomilast (CLM), and PDE4 activity was measured as described in the Methods section. PDE4 activity was expressed as fmol·min^{-1}·10^6 cells^{-1} and data are shown as mean±SEM for three independent experiments. ***: $p<0.001$ for unstimulated PMN *versus* EOS.

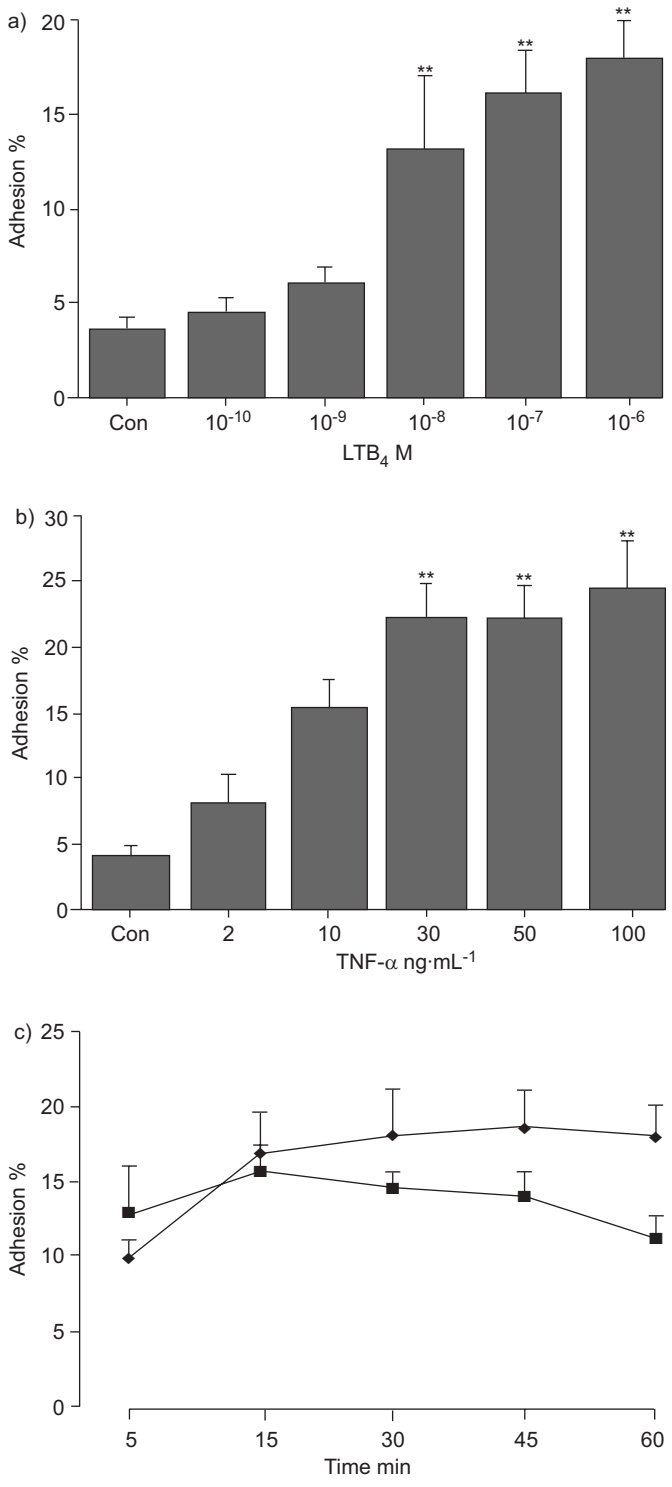

FIGURE 3. Kinetics and concentration-dependent effect of leukotriene (LT)B_4 and tumour necrosis factor (TNF)-α on adhesion. Polymorphonuclear leukocytes (PMNs) were activated with increasing concentrations of a) LTB_4 for 15 min (**: $p<0.01$ for $>10^{-9}$ M LTB_4 *versus* control) or b) TNF-α for 30 min prior to adhesion assay (**: $p<0.01$ for >10 ng·mL^{-1} TNF-α *versus* control). c) Time course for adhesion for TNF-α- or LTB_4-activated PMNs. Untreated PMNs were added to bovine serum albumin-coated wells and activated with optimal concentration of LTB_4 (■; 10^{-7} M) or TNF-α (◆; 30 ng·mL^{-1}) at various times. Adhesion was measured as a function of residual myeloperoxidase activity. Data are expressed as mean±SEM for six independent experiments.

was comparable, as assayed by measuring the residual MPO after activation with FMLP (fig. 1). Pre-incubation of PMNs with anti-CD11b (the α-chain of Mac-1, which is present on PMN) or anti-CD18 (the common β_2-chain) specifically and equivalently blocked adhesion to BSA and ICAM-1. Neither mAb directed against CD11a (the α-chain of leukocyte function-associated antigen-1) nor CD49d (the α-chain of very late antigen-4, which is not present on PMN) blocked adhesion caused by FMLP. These data demonstrate that the pattern of ligation of BSA is similar to ICAM-1 and thus is suitable for measurement of β_2-integrin adhesion. Accordingly, for all subsequent experiments, BSA was used as a surrogate protein for ICAM-1 [24, 27, 28].

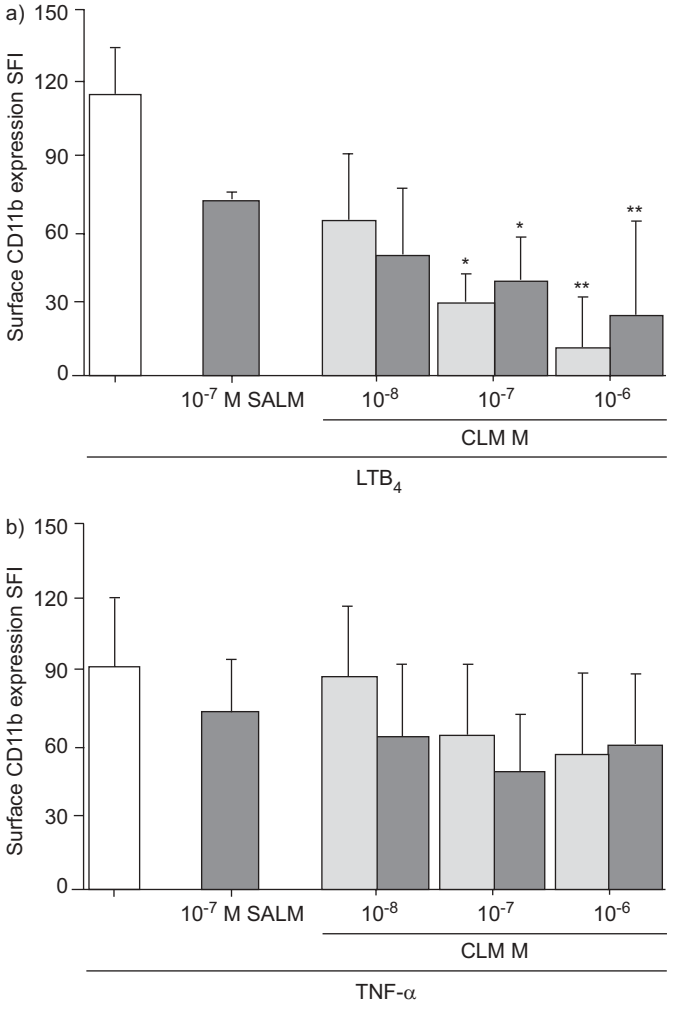

FIGURE 4. Effect of cilomilast (CLM) and/or salmeterol (SALM) on surface expression of CD11b. Polymorphonuclear leukocytes (PMNs) were pre-incubated with either 10^{-8}–10^{-6} M CLM alone (▒), 10^{-7} M SALM alone, or CLM+10^{-7} M SALM (■) prior to activation with a) 10^{-7} M leukotriene (LT)B_4 for 15 min or b) 30 ng·mL^{-1} tumour necrosis factor (TNF)-α for 30 min at 37°C. Surface CD11b expression was analysed by flow cytometry and data are expressed as specific fluorescence intensities (SFI; mean fluorescence intensity minus unstimulated control). *: $p<0.05$ for LTB_4-activated PMNs (no CLM, no SALM) *versus* 10^{-7} M CLM+LTB_4 with or without SALM; **: $p<0.01$ for 10^{-6} M CLM+LTB_4 with or without SALM. p=NS for TNF-α-activated PMNs *versus* all treatments and concentrations.

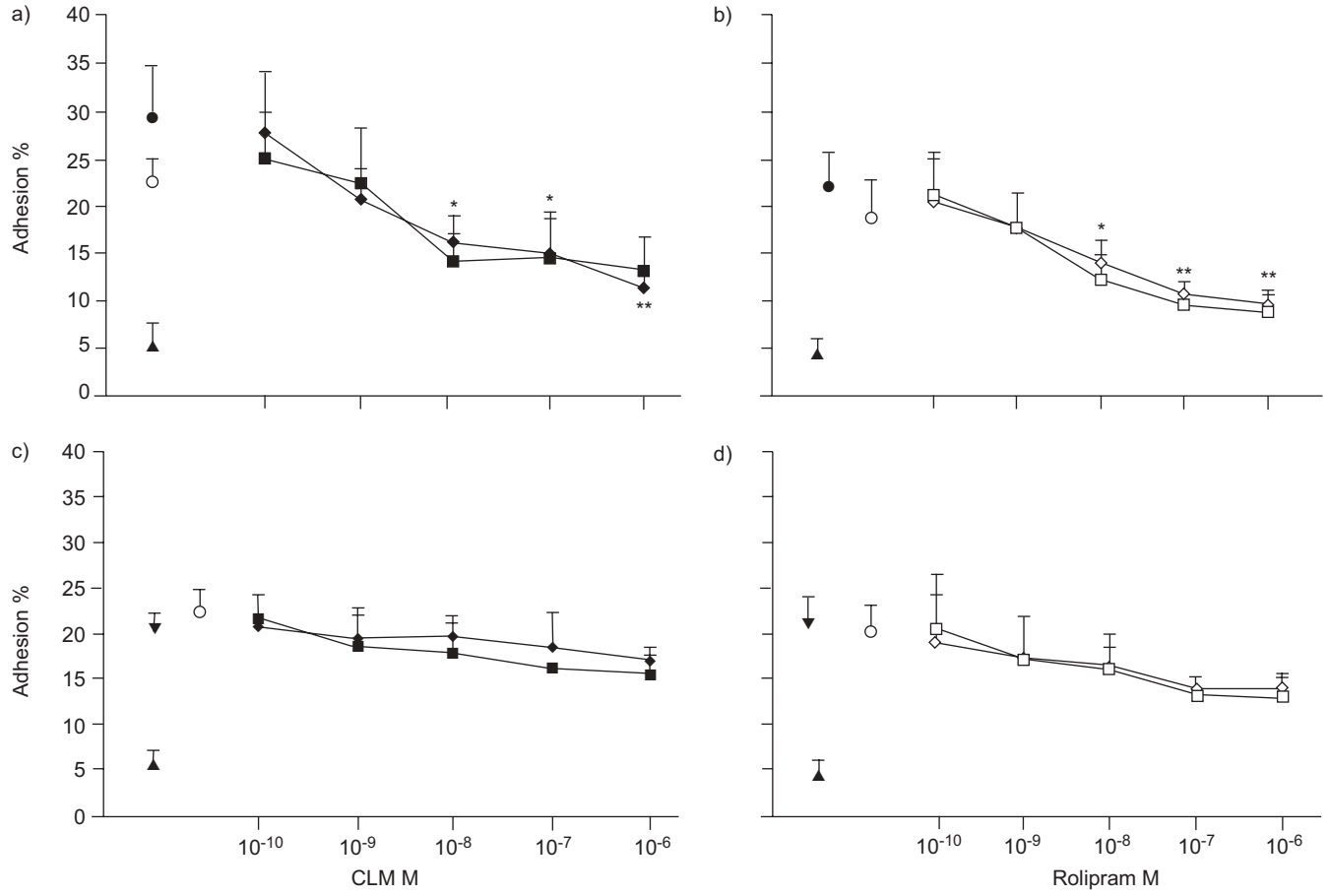

FIGURE 5. Effect of phosphodiesterase (PDE)4 inhibitors (cilomilast (CLM) or rolipram) and/or salmeterol (SALM) on stimulated adhesion. Polymorphonuclear leukocytes (PMNs) were pre-incubated with either a) CLM and/or SALM or b) rolipram and/or SALM prior to activation with 10^{-7} M leukotriene (LT)B_4 (a and b) for 15 min or 30 ng·mL^{-1} tumour necrosis factor (TNF)-α (c and d) for 30 min at 37°C. Adhesion was measured as a function of residual myeloperoxidase activity. Data are expressed as mean±SEM for six independent experiments. ●: LTB_4; ○: SALM; ▲: control; ♦: CLM alone; ■: CLM+10^{-7} M SALM; ▼: TNF-α; ◇: rolipram alone; □: rolipram+10^{-7} M SALM.

PDE4 activity: PMNs versus eosinophils

As a reference, PDE4 activity in both freshly isolated PMNs and eosinophils was measured. It was found that the PDE4 activity in quiescent PMNs was ~10-fold greater than the PDE4 activity in human eosinophils (fig. 2). PDE4 activity in PMNs was 192±96.3 fmol·min^{-1}·10^6 cells^{-1} *versus* 16.2±10.0 fmol·min^{-1}·10^6 cells^{-1} for eosinophils ($p<0.001$). However, 10^{-6} M cilomilast, the greatest concentration used in subsequent studies, completely inhibited PDE4 activity in both PMNs and eosinophils.

Kinetics and concentration-dependent effect of LTB_4 and TNF-α on β_2-integrin-mediated adhesion

The kinetics of PMN adhesion to BSA-coated microplate wells in response to LTB_4 and TNF-α activation were then determined. Adhesion caused by LTB_4 (fig. 3a) and TNF-α (fig. 3b) increased in a concentration-dependent manner. Maximal adhesion occurred at 15 min for cells activated with 10^{-7} M LTB_4 and at ⩾25 min after activation with 30 ng·mL^{-1} of TNF-α (fig. 3c). These times and concentrations for LTB_4 and TNF-α were used for all subsequent experiments.

Effect of LTB_4 and TNF-α on CD11b surface expression: blockade with cilomilast and/or salmeterol

The effect of LTB_4 (fig. 4a) and TNF-α (fig. 4b) on the surface expression of CD11b on human PMNs was examined further. Stimulation with LTB_4 increased the surface CD11b expression to 116±18 specific fluorescence intensities (SFI; $p<0.05$ *versus* unstimulated cells) and 91.8±29 SFI for cells activated with TNF-α ($p<0.05$ *versus* unstimulated cells). Pre-treatment of PMNs with 10^{-7} M cilomilast attenuated the CD11b expression to 29.4±12 SFI ($p<0.05$ *versus* LTB_4-activated cells) and further to 11.7±21 SFI for cells pre-treated with 10^{-6} M cilomilast ($p<0.01$ *versus* LTB_4-activated cells). By contrast, cilomilast did not significantly affect the upregulated CD11b expression caused by TNF-α stimulation at any concentrations (fig. 4b). Neither salmeterol alone nor a combination of cilomilast and salmeterol had an additive inhibitory effect on stimulated CD11b expression in PMNs.

Blockade of adhesion caused by LTB_4 or TNF-α

Subsequently, the effect of LTB_4 and TNF-α on β_2-integrin-mediated adhesion to BSA-coated microplate wells before and

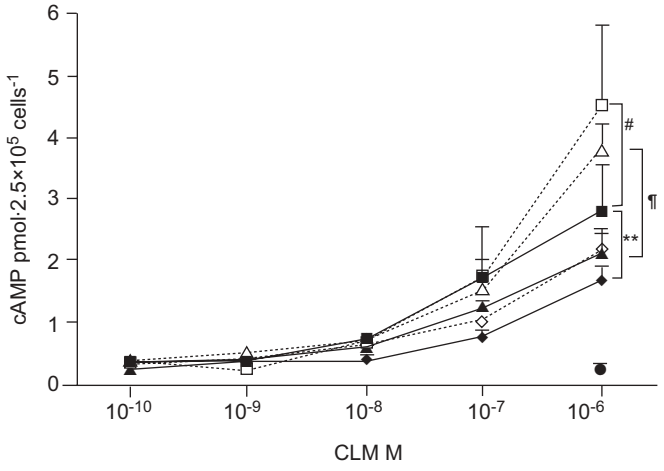

FIGURE 6. Effect of cilomilast (CLM) and/or salmeterol (SALM) on intracellular cyclic adenosine monophosphate (cAMP) concentration. Polymorphonuclear leukocytes (PMNs) were pre-incubated with either 10^{-10}–10^{-6} M CLM alone (♦) or CLM+10^{-7} M SALM prior to activation with 10^{-7} M leukotriene (LT)B_4 for 15 min or 30 ng·mL^{-1} tumour necrosis factor (TNF)-α for 30 min at 37°C and measurement of intracellular cAMP concentration. □: CLM+SALM+LTB_4; △: CLM+SALM+TNF-α; ■: CLM+LTB_4; ▲: CLM+TNF-α; ◇: CLM+SALM; ●: SALM alone. **: $p<0.01$ for 10^{-6} M CLM+LTB_4-activated PMNs *versus* 10^{-6} M CLM-alone-treated PMNs; #: $p<0.01$ for PMNs treated with 10^{-6} M CLM+10^{-7} M SALM+LTB_4 *versus* 10^{-6} M CLM+LTB_4, no SALM; ¶: $p<0.01$ for PMNs treated with 10^{-6} M CLM+10^{-7} M SALM+TNF-α *versus* 10^{-6} M CLM+TNF-α, no SALM. Data (n=4) are expressed as mean±SEM in pmol·2.5×10^5 cells^{-1} .

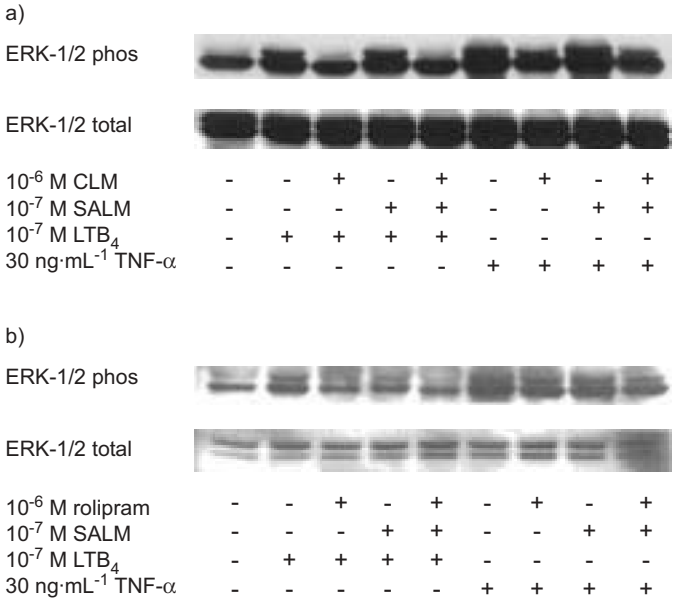

FIGURE 7. Inhibitory effect of phosphodiesterase 4 inhibitors (cilomilast (CLM) or rolipram) and/or salmeterol (SALM) on extracellular signal-regulated kinase (ERK)-1/2 phosphorylation (phos). A representative ERK-1/2 phos caused by leukotriene (LT)B_4 or tumour necrosis factor (TNF)-α-stimulated polymorphonuclear leukocytes (PMNs) in the presence or absence of 10^{-6} M cilomilast (a) or rolipram (b) and/or 10^{-7} M SALM. Treated PMNs were lysed and subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by immunoblotting analysis using anti-phosphorylation specific ERK-1/2 antibody. Equal sample loading was confirmed by using total anti-ERK-1/2 antibody.

after exposure to cilomilast and/or salmeterol was examined. Adhesion caused by LTB_4 activation was 29.4±5.3% and decreased to 16.2±2.8% for cells treated with 10^{-8} M cilomilast (fig. 5a; $p<0.05$), to 15.0±4.27% after 10^{-7} M cilomilast ($p<0.05$ *versus* LTB_4-activated cells), and to 11.3±2.2% after 10^{-6} M cilomilast ($p<0.01$ *versus* LTB_4-activated cells). Salmeterol alone caused no blockade of β_2-integrin-mediated adhesion. The addition of salmeterol to cilomilast caused no augmentation (*versus* cilomilast alone) in the blockade of β_2-integrin-mediated adhesion caused by LTB_4 at any concentration (fig. 5a).

Maximal adhesion caused by TNF-α was nearly comparable to that elicited by LTB_4 (20.5±1.5%). In contrast to LTB_4, however, pre-treatment with cilomilast alone or salmeterol+cilomilast had no inhibitory effect on PMN adhesion to ICAM-1 surrogate protein for PMN activated by TNF-α (fig. 5b).

The same experiments were repeated with different PMNs treated with either rolipram alone or rolipram+salmeterol. Results were comparable to those obtained with cilomilast or cilomilast+salmeterol (fig. 5c and d).

Effect of cilomilast and salmeterol on stimulated intracellular cAMP concentration

To assess the potential relationship between the PDE4/β_2-adrenoceptor inhibition on stimulated adhesion, concentrations of cAMP were analysed for stimulated cells in the presence of cilomilast alone, salmeterol alone, and cilomilast+salmeterol. Baseline intracellular cAMP concentration (before cilomilast±salmeterol) was insignificant for all treated groups. Intracellular cAMP was 0.19±0.04 pmol·2.5×10^5 cells^{-1} for buffer alone, 0.29±.04 pmol·2.5×10^5 cells^{-1} after treatment with LTB_4 alone and 0.14±.01 pmol·2.5×10^5 cells^{-1} for PMNs activated with TNF-α ($p=$ NS for all comparisons). The addition of cilomilast increased intracellular cAMP in a concentration-dependent manner (fig. 6). At 10^{-8} M cilomilast, cAMP concentration was 0.38±0.08 pmol·2.5×10^5 cells^{-1}; after 10^{-7} M cilomilast it was 0.78±0.10 pmol·2.5×10^5 cells^{-1} ($p<0.05$ *versus* buffer control); and for PMNs treated with 10^{-6} M cilomilast it was 1.69±0.24 pmol·2.5×10^5 cells^{-1} ($p<0.01$ *versus* buffer control). By contrast, exposure to 10^{-7} M salmeterol alone caused no increase in intracellular cAMP ($p=$NS).

Co-incubation of salmeterol with 10^{-10}–10^{-6} M cilomilast caused an additive increase in intracellular cAMP concentrations. Pre-treatment with $\geq 10^{-8}$ M cilomilast alone or cilomilast+salmeterol augmented the increase of the cAMP concentrations in PMNs subsequently treated with LTB_4 or TNF-α. For PMN treated with 10^{-6} M cilomilast+LTB_4, cAMP increased by 1.6-fold to 2.8±0.73 pmol·2.5×10^5 cells^{-1} ($p<0.01$ *versus* 10^{-6} M cilomilast alone; fig. 6). Pre-incubation of PMNs with salmeterol+10^{-6} M cilomilast+LTB_4 further augmented intracellular cAMP concentration to 4.5±1.3 pmol·2.5×10^5 cells^{-1}, an ~1.6-fold increase from 10^{-6} M cilomilast+LTB_4 without salmeterol ($p<0.01$).

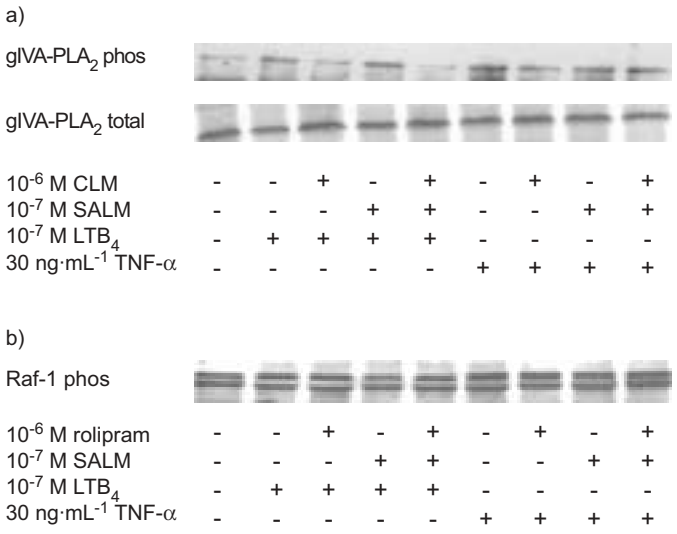

FIGURE 8. Effects of cilomilast (CLM) and/or salmeterol (SALM) on group IVA-phospholipase A_2 (gIVA-PLA_2) and Raf-1 phosphorylation (phos). A representative a) gIVA-PLA_2 phosphorylation and b) Raf-1 phosphorylation caused by leukotriene (LT)B_4 or tumour necrosis factor (TNF)-α-stimulated polymorphonuclear leukocytes (PMNs) in the presence or absence of 10^{-6} M CLM and/or 10^{-7} M SALM. Treated PMNs were lysed and subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by immunoblotting analysis using anti-phosphorylation Ser505-specific gIVA-PLA_2 antibody (Ab) (a) and anti-phosphorylation Ser259 Raf-1 Ab (b). Equal sample loading was confirmed by using total anti-gIVA-PLA_2 Ab.

Activation of PMNs with cilomilast+TNF-α caused comparable increase in cAMP concentration when compared with PMN treated with cilomilast+salmeterol or cilomilast+LTB_4 ($p=$NS). Salmeterol+cilomilast+TNF-α increased the cAMP level from 2.1 ± 0.36 pmol·2.5×10^5 $cells^{-1}$ for PMNs treated with 10^{-6} M cilomilast+TNF-α without salmeterol to 3.8 ± 0.40 pmol·2.5×10^5 $cells^{-1}$ ($p<0.01$).

Activation of ERK-1/2 and gIVA-PLA_2 by LTB_4 and TNF-α: effect of cilomilast and/or salmeterol

The next step was to determine whether LTB_4-mediated adhesion of β_2-integrin on PMNs was mediated through the ERK-1/2 and gIVA-PLA_2 pathways. Activation of cells with LTB_4 caused activation of ERK-1 and gIVA-PLA_2 phosphorylation, which was blocked by 10^{-6} M cilomilast, as assessed by Western blot analysis (figs 7 and 8a). Co-incubation of PMNs with salmeterol either alone or in combination with cilomilast caused no further inhibition of ERK-1 and gIVA-PLA_2 phosphorylation caused by LTB_4. ERK-2 was constitutively phosphorylated, even in the resting state (fig. 7). Significant upregulation of phosphorylated ERK-2 was not established after activation with either LTB_4 or TNF-α. An identical pattern of blockade of ERK-1/2 phosphorylation was demonstrated with either cilomilast or rolipram alone or in combination with salmeterol (fig. 7).

In additional experiments, the effect of TNF-α on ERK-1/2-mediated phosphorylation of gIVA-PLA_2 was examined. Like LTB_4, TNF-α caused phosphorylation of ERK-1 and downstream gIVA-PLA_2; however, treatment with cilomilast alone and/or salmeterol did not effectively block ERK-1 or gIVA-PLA_2 phosphorylation caused by TNF-α activation. Hence, while PMNs pre-treated with cilomilast+β_2-agonist demonstrated a comparable and substantial increase in intracellular cAMP after treatment with LTB_4 or TNF-α, downstream inhibition of ERK and gIVA-PLA_2 phosphorylation was prevented by treatment with TNF-α. These data indicated that TNF-α prevents the blockade of essential ERK-mediated pathways necessary to translate cAMP-mediated signalling into blockade of β_2-integrin adhesion.

A final series of experiments tested the hypothesis that cAMP activation caused by TNF-α was not effective in blocking adhesion because TNF-α simultaneously blocked Raf-1 phosphorylation (fig. 8b). Raf-1 was present for both LTB_4- and TNF-α-activated neutrophils and was phosphorylated comparably in both groups after treatment with cilomilast.

DISCUSSION

The objectives of the current study were to determine the following: 1) whether blockade of cAMP degradation by PDE4 inhibition would cause a blockade of β_2-integrin-mediated adhesion; and 2) the signalling pathways involved in cAMP-mediated upregulation of integrin adhesion in PMN.

PDE4 inhibitor caused a concentration-related inhibitory effect on adhesion for PMN stimulated with LTB_4, but not with the cytokine TNF-α. Inhibition of integrin adhesion caused by LTB_4 was not further augmented by β_2-adrenoceptor stimulation (fig. 5), despite augmented production of intracellular cAMP (fig. 6).

Data from the present study indicate that PMNs express substantially greater concentrations of PDE4 than eosinophils (fig. 2) in the same human donors. The inhibitory effect of cilomilast, either alone or in combination with salmeterol, on β_2-integrin-mediated adhesion caused by two agonists, LTB_4 and TNF-α in human PMNs, was also investigated. It was found that cilomilast, a specific PDE4 inhibitor is responsible for blocking of PDE4 activity in resting granulocytes (fig. 2) and increasing intracellular cAMP concentration (fig. 6); it thus inhibits ERK-1-mediated gIVA-PLA_2 phosphorylation caused by LTB_4 activation in PMNs (fig. 8). Inhibition of gIVA-PLA_2 phosphorylation blocked expression of upregulated surface CD11b (fig. 4) and prevented adhesion mediated by β_2-integrin (fig. 5).

The present data indicate that this blocking effect of cilomilast$\pm$salmeterol on β_2-integrin-mediated adhesion for LTB_4-activated PMN corresponds to a comparable blockade of cell surface CD11b expression (fig. 4) and to ERK-1-mediated gIVA-PLA_2 phosphorylation (fig. 8). Interestingly, ERK-2, which is constitutively expressed, does not appear to regulate PMN adhesion (fig. 7).

PMN activated by TNF-α had β_2-integrin adhesion comparable to that for LTB_4, and had a comparable increase in intracellular cAMP after treatment with cilomilast, but little or no inhibition of ERK-1 or gIVA-PLA_2 phosphorylation (figs 7 and 8a). Accordingly, these data suggest that TNF-α prevents inhibition of cAMP-mediated activation of ERK-1, which further prevents downstream inhibition of gIVA-PLA_2 phosphorylation. The

current authors have previously shown that gIVA-PLA_2 phosphorylation is an essential step in integrin adhesion [29]. Thus, the ability of TNF-α to block inhibition of gIV-PLA_2 phosphorylation confers unique resistance of this cytokine to blocking attenuation of β_2-integrin adhesion.

It is important to consider some limitations of the present findings. The data presently obtained, while using human cells, could only be obtained *in vitro*. The direct interaction between β_2-agonist and PDE4 inhibitor *in vivo* cannot be assessed precisely. Nonetheless, the present data indicate substantial differences in the ability of PDE4 inhibitor to block TNF-α- and LTB_4-induced adhesion of β_2-integrin on PMNs. In the present investigation, the effects of PDE4 inhibitor$\pm$ salmeterol on stimulated ERK-1/2, which the present authors have previously shown to cause phosphorylation of gIVA-PLA_2 in human eosinophils, were examined [17]. The present data clearly demonstrate a direct relationship between inhibition by PDE4 of ERK-1 phosphorylation and β_2-integrin adhesion for PMN activated by LTB_4, but this is not so for TNF-α. Nevertheless, the present study did not explore all possible inhibitory pathways. Specifically, the potential role of p38 mitogen-activated protein kinase was not examined, as this isoform is constitutively expressed in its phosphorylated state and does not regulate β_2-integrin in eosinophils [30]. It is likely that other inhibitory pathways may also be active in regulating β_2-integrin, *e.g.* phosphatidylinositide 3-kinase. In prior studies, G-protein-mediated adhesion has been shown to be regulated by ERK-1/2–gIVA-PLA_2 interaction [18, 30]. In the present study, a definite relationship has been shown between ERK-1/2- and gIVA-PLA_2-induced adhesion caused by LTB_4. Blockade of this phosphorylation by PDE4 inhibition also blocked adhesion. Clearly, there is a limitation to the number of combinations of experiments that can be performed in a single study. However, the present data demonstrate that the ability to block integrin adhesion is stimulus dependent and corresponds directly to ERK-1-mediated gIVA-PLA_2 phosphorylation. It should be noted that the therapeutic efficacy of PDE4 inhibition of PMN adhesion and transendothelial migration cannot be predicted from the present studies. However, TNF-α-induced resistance to downregulation of adhesion caused by cAMP-mediated mechanisms indicates that the circumstances of activation may, in a large part, determine the efficacy of anti-neutrophilic therapies.

In summary, it was found that tumour necrosis factor-α prevents cyclic adenosine monophosphate-induced inhibition of extracellular signal-regulated kinase-1 phosphorylation, which further prevents blockade of β_2-integrin adhesion. By contrast, leukotriene B_4-activated polymorphonuclear leukocytes with comparable increase in cyclic adenosine monophosphate elicited by cilomilast demonstrated both inhibition of extracellular signal-regulated kinase-1 and extracellular signal-regulated kinase-1/2-mediated group IVA-phospholipase A_2 phosphorylation, and blockade of β_2-integrin adhesion. The mechanism by which extracellular signal-regulated kinase-1/2-mediated group IVA-phospholipase A_2 causes β_2-integrin adhesion remains undefined. Accordingly, while tumour necrosis factor-α activated cells have been identified as being refractory to cyclic adenosine monophosphate-initiated blockade of β_2-integrin adhesion, the precise mechanism by which this occurs cannot yet be defined. These data nonetheless suggest that blockade of β_2-integrin adhesion in polymorphonuclear leukocytes depends critically upon the mode of activation.

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