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PDE4-inhibition on β_2-integrin Adhesion Caused by LTB_4 and TNFα in Human Neutrophils

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ABSTRACT

Phosphodiesterase (PDE)4-inhibition attenuates neutrophilic inflammation in chronic obstructive pulmonary disease.

The objective of this study was to examine the efficacy and mechanism by which PDE4-inhibition blocks adhesion of β_2-integrin to endothelial counterligand.

Neutrophils (PMN)s were isolated from humans receiving no medications. Adhesion was analyzed by myeloperoxidase activity. The effects of cilomilast ± salmeterol on a) surface CD11b expression, b) adhesion, c) intracellular cAMP concentration, and d) ERK-1/2 mediated group IVA-phospholipase A_2 (gIVA-PLA_2) phosphorylation caused by LTB_4 or TNFα activation were determined.

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

We demonstrate that both LTB_4 and TNFα upregulate cAMP. However cAMP does not block β_2-integrin adhesion caused by TNFα. We conclude that TNFα *prevents* inhibition of gIVA-PLA_2 activation, which is essential for β_2-integrin adhesion in PMN.

Key Words: phosphodiesterase-4, inflammation, adhesion, neutrophils

Introduction

Cyclic nucleotide phosphodiesterases (PDEs) are 11 families of PDE enzymes (PDE1-PDE11), eight of which generate over 30 different isoforms able to metabolize and inactivate the naturally occurring second messenger nucleotide, 3',5'-cyclic monophosphates (cAMP) (1-3). Among the isoforms, PDE4 enzymes selectively hydrolyze cAMP and have a low affinity for cGMP (1-3). Because of 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, 4 genotypes for PDE4 have been identified (PDE4A-PDE4D); PDE4A, PDE4B and PDE4D are particularly abundant in several inflammatory cells including, neutrophils (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 established fully.

Guinea pig peritoneal eosinophils express predominantly a membrane bound PDE4, while human eosinophils express PDE4 and PDE7 as intracellular isoenzymes (12). PDE isoenzymes in human PMNs have been incompletely characterized; 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)**.**

The inhibitory effects of PDE4 on down-regulation of neutrophil and eosinophil functions, i.e. cellular adhesion, granule secretion, CD11b expression, shedding of L-Selectin, have been reported previously (5, 13). PDE4A is localized within cytoplasmic granules of granulocytes and predominantly translocated to the plasmalemma in response to FMLP activation (5); a small amount of is some PDE4 was exocytosed in the activated state (5). Studies in vivo have demonstrated the inhibitory effect of rolipram on endothelial transmigration

of neutrophils and eosinophils (14, 15). Other studies have show that inhibition of PDE4 causes relaxation of hyperreactive airway smooth muscle and blockade of mediator release by inflammatory cells (15, 16).

Recently, we demonstrated that rolipram, a PDE4 inhibitor, blocks the synthesis of cysteinyl LTC_4 by ~50% in eosinophils activated by formyl-met-leu-phe (FMLP) (17). Co-incubation with salmeterol caused synergistic inhibition of this response (17). We also have demonstrated that rolipram blocks β_2-integrin mediated adhesion caused by eotaxin in human eosinophils, but not IL-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 sensitized 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 this study, we examined the comparative inhibitory effect of PDE4 inhibition using cilomilast alone and cilomilast in combination with salmeterol on β_2-integrin mediated adhesion caused by TNFα or LTB_4 in human PMNs in vitro. We find that β_2-integrin adhesion depends critically upon the upstream stimulus activating gIVA-PLA_2 phosphorylation.

Methods

Isolation of eosinophils and PMNs

Eosinophils were isolated from 18 human subjects (20-45 years old) as previously described (23). Informed written consent was obtained from all volunteers. None of the subjects had received any medication for at least 4 weeks before the study.

Eosinophils were isolated by immunomagnetic depletion of PMNs using anti-CD16–coated MACS particles (Miltenyi Biotec, Sunnyvale, CA). Purity was ≥ 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; Amersham). The cell pellet containing PMNs was resuspended in HBSS buffer + Ca^{++}/0.2% 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.

a) Measurement of PMN adhesion: verification of BSA-surrogate

Microplate wells were coated with 50 μl of either soluble 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 [Bradley, 1982 #66]. PMNs (4×10^4/100 μl HBSS/0.1% gelatin) 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. Fifteen min later, microplate wells were gently washed with HBSS, and 100 μl of HBSS/0.1% gelatin was added to the wells. 100 μl MPO substrate (0.01% H_2O_2, 0.167 mg/ml *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 mixture was terminated. 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). 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 TNFα were generated for subsequent studies (see below). These concentrations of LTB_4 and TNFα were those approximating maximal β_2-integrin adhesion (see Results).

b) Blockade of stimulated β_2-integrin mediated adhesion.

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

Determination of PDE4 activity

PDE4 activity in quiescent PMNs and eosinophils was analyzed by a modified method of Thompson and Appleman (25, 26). Cells (2 x 10^6 cells) were treated with buffer alone or 10^{-6} M cilomilast alone (30 min). The enzyme containing fractions were assayed in a final volume of 200 µl containing 0.5µM cyclic AMP (28000 cpm [^{3}H]-cAMP) and incubated for 30 min at 37^{o}C. The reaction mixture was terminated by addition of 50 µl of 0.2 N 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 scintillation cocktail, and radioactivity was counted using a Beckman liquid scintillation counter. PDE4 activity was expressed as fentamole per minute per million cells (fmol/min/10^6 cells).

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

PMNs were preincubated with 10^{-10} to 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 TNFα (30 min) and addition of 300 µl cold FACS buffer (PBS with1% BSA and 0.1% NaN_3). The cell pellet was washed once with FACS buffer prior to incubation with 4 µl CD11b mAb (Endogen, Woburn, MA), or isotype-matched control Ab at 4°C for 60 min. After 2X washing, cells were incubated with excess of FITC-conjugated goat anti-mouse 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) 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 preincubated 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 TNFα, or buffer control at 37°C. Adhesion assay was performed as above.

Determination of intracellular cAMP concentration

cAMP was assay by EIA (Assay Design, Ann Arbor, MI). PMNs (2.5 x 10^5 cells/intervention) were preincubated with 10^{-10} M to 10^{-6} M cilomilast and/or 10^{-7} M salmeterol prior to activation with 10^{-7} M LTB_4, 30 ng/ml TNFα, 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 picomole per 2.5 x 10^5 cells (pmol/2.5 x 10^5 cells).

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

PMNs were preincubated with either 10^{-10} M to 10^{-6} M cilomilast or rolipram and/or 10^{-7} M salmeterol prior to activation with 10^{-7} M LTB_4, 30 ng/ml TNFα, 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 of 6x sample buffer and boiled for 5 min. Samples were loaded to SDS-PAGE 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 TBS-T buffer for 60 min prior to addition of 2μg/ml anti-phosphorylated ERK-1/2 Ab (Promega, Madison, WI) or 2μg/ml anti-phosphorylated gIVA-PLA_2 Ab (Ser^{505}, Cell Signaling Technology, Beverly, MA) or 2μg/ml anti-phospho-Raf Ab (Ser^{259}, Cell Signaling Technology, Beverly, MA). After washing, the membranes were incubated with 1:3000 dilution of goat anti-rabbit Ig conjugated with HRP and analyzed by an enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

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 on concentration–response curves for the same agonist or inhibitor were compared after Bonferonni correction. Variation among more than two groups was tested using ANOVA followed by Fisher's least protected difference test. Statistical significance was claimed when $p < 0.05$.

RESULTS

Equivalency of binding of ICAM-1 and BSA Surrogate

a) 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, we compared the adhesion of PMNs to BSA as a surrogate protein for the immunoglobulin supergene. The number of PMNs adhering to ICAM-1 or BSA-coated wells was comparable as assayed by measuring the residual MPO after activation with FMLP (Figure 1). Preincubation 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 LFA-1) nor CD49d (the α-chain of VLA-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 surrogate protein for ICAM-1 (24, 27, 28).

b) PDE4 activity: PMNs versus Eosinophils

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

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

We next determined the kinetics of PMN adhesion to BSA-coated microplate wells in response to LTB_4 and TNFα activation. Adhesion caused by LTB_4 (Figure 3A) and TNFα (Figure 3B) increased in concentration-dependent manner. Maximal adhesion occurred at 15 min for cells activated with 10^{-7} M LTB_4 and $\geq$ 25 min after 30 ng/ml TNFα activation (Figure 3C). These times and concentrations for LTB_4 and TNFα were used for all subsequent experiments.

Effect of LTB_4 and TNFα on CD11b surface expression: blockade with cilomilast and/or salmeterol

We further examined the effect of LTB_4 (Figure 4A) and TNFα (Figure 4B) on the surface expression of CD11b on human PMNs. Stimulation with LTB_4 increased the surface CD11b expression to 116 ± 18 specific fluorescence intensity (SFI; $P < 0.05$ versus unstimulated cells) and 91.8 ± 29 SFI for cells activated with TNFα ($P < 0.05$ versus unstimulated cells). Pretreatment 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 pretreated with 10^{-6} M cilomilast ($P < 0.01$ versus LTB_4-activated cells). By contrast, cilomilast did not affect significantly the upregulated CD11b expression caused by TNFα stimulation at any concentrations (Figure 4B). Neither salmeterol alone nor combination of cilomilast and salmeterol had an additive inhibitory effect on stimulated CD11b expression in PMNs.

Blockade of Adhesion caused by LTB_4 or TNFα

We next examined the effect of LTB_4 and TNFα on β_2-integrin mediated adhesion to BSA-coated microplate wells before and after exposure to cilomilast and/or salmeterol. Adhesion caused by LTB_4 activation was 29.4 ± 5.3% and decreased 16.2 ± 2.8% for cells treated with 10^{-8} M cilomilast (Figure 5A; $P < 0.05$), 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 (Figure 5A).

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

The same experiments were repeated with different PMN treated with either rolipram alone or rolipram + salmeterol. Results were comparable to those obtained with cilomilast or cilomilast + salmeterol (Figure 5C, 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 analyzed 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 x 10^5 cells for buffer alone, 0.29 ± .04 pmol/2.5 x

10^5 cells after treatment with LTB_4 alone and 0.14 ± .01 pmol/2.5 x 10^5 cells for PMNs activated with TNFα (p = NS for all comparisons). The addition of cilomilast increased intracellular cAMP in concentration-dependent manner (Figure 6). At 10^{-8} M cilomilast, cAMP concentration was 0.38 ± 0.08 pmol/2.5 x 10^5 cells, 0.78 ± 0.10 pmol/2.5 x 10^5 cells after 10^{-7} M cilomilast ($P < 0.05$ versus buffer control) and 1.69 ± 0.24 pmol/2.5 x 10^5 cells for PMNs treated with 10^{-6} M cilomilast ($P < 0.01$ versus buffer control). By contrast, exposure to 10^{-7} M salmeterol alone did not cause any increase in intracellular cAMP (P = NS).

Co-incubation of salmeterol with 10^{-10} M to 10^{-6} M cilomilast caused an additive increase in intracellular cAMP concentrations. Pretreatment with $\geq 10^{-8}$ M cilomilast alone or cilomilast + salmeterol augmented the increase of the cAMP concentrations in PMN subsequently treated with LTB_4 or TNFα. For PMN treated with 10^{-6} M cilomilast + LTB_4, cAMP increased by 1.6-fold to 2.8 ± 0.73 pmol/2.5 x 10^5 cells ($P < 0.01$ versus 10^{-6} M cilomilast alone) (Figure 6). Preincubation of PMNs with salmeterol + 10^{-6} M cilomilast + LTB_4 further augmented intracellular cAMP concentration to 4.5 ± 1.3 pmol/2.5 x 10^5 cells, an approximate 1.6-fold increase from 10^{-6} M cilomilast + LTB_4 without salmeterol ($P < 0.01$).

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

Activation of ERK-1/2 and gIVA-PLA$_2$ by LTB$_4$ and TNFα: effect of cilomilast and/or salmeterol

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 (Figure 7, 8A). Co-incubation of PMNs with salmeterol 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 (Figure 7). Significant upregulation of phosphorylated ERK-2 was not established after activation with either LTB$_4$ or TNFα. An identical pattern of blockade of ERK1/2-phosphorylation was demonstrated with either cilomilast or rolipram alone or in combination with salmeterol (Figure 7).

In additional experiments, we further examined the effect of TNFα on ERK-1/2 mediated phosphorylation of gIVA-PLA$_2$. Like LTB$_4$, TNFα 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 TNFα activation. Hence, while PMNs pretreated with cilomilast + β$_2$-agonist demonstrated comparable and substantial increase in intracellular cAMP after LTB$_4$ or TNFα, downstream inhibition of ERK and gIVA-PLA$_2$ phosphorylation was prevented by treatment with TNFα. These data indicated that TNFα prevents the blockade of essential ERK mediated pathways necessary to translate cAMP-mediated signaling into blockade of β$_2$-integrin adhesion.

In a final series of experiments, we tested the hypothesis that cAMP activation caused by TNFα was not effective in blocking adhesion because TNFα simultaneously blocked Raf-1

phosphorylation (Fig 8B). Raf-1 was present for both LTB_4- and TNFα- activated neutrophils and was phosphorylated comparably in both groups after treatment with cilomilast.

Discussion

The objectives of our study were to determine a) whether blockade of cAMP degradation by PDE4 inhibition would cause blockade of β_2-integrin mediated adhesion and b) the signaling 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, TNFα. Inhibition of integrin adhesion caused by LTB_4 was not further augmented by β_2-adrenoceptor stimulation (Figure 5), despite augmented production of intracellular cAMP (Figure 6).

Our data indicate that PMNs express substantially greater concentrations of PDE4 than eosinophils (Figure 2) in the same human donors. We also investigated the inhibitory effect of cilomilast alone or in combination with salmeterol on β_2-integrin mediated adhesion caused by two agonists, LTB_4 and TNFα in human PMNs. We found that cilomilast, a specific PDE4 inhibitor, a) blocks PDE4 activity in resting granulocytes (Figure 2), b) causes increased intracellular cAMP concentration (Figure 6), and c) thus inhibits ERK-1 mediated gIVA-PLA_2 phosphorylation caused by LTB_4 activation in PMNs (Figure 8). Inhibition of gIVA-PLA_2 phosphorylation blocked upregulated surface CD11b expression (Figure 4) and prevented adhesion mediated by β_2-integrin (Figure 5).

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

PMN activated by TNFα had comparable β_2-integrin adhesion as for LTB_4 and comparable increase in intracellular cAMP after cilomilast, but little or no inhibition of ERK-1 or gIVA-PLA_2 phosphorylation (Figure 7, 8A). Accordingly, these data suggest that TNFα prevents inhibition of cAMP-mediated activation of ERK-1, which further prevents downstream inhibition of gIVA-PLA_2 phosphorylation. We have shown previously that gIVA-PLA_2 phosphorylation is an essential step in integrin adhesion (29). Thus, the ability of TNFα to block inhibition of gIV-PLA_2 phosphorylation confers unique resistance of this cytokine to block attenuation of β_2-integrin adhesion.

It is important to consider some limitations of our findings. These data, 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, our data indicate substantial differences in the ability of PDE4 inhibitor to block TNFα- and LTB_4-induced adhesion of β_2-integrin on PMNs. In this investigation, we examined the effects of PDE4 inhibitor ± salmeterol on stimulated ERK-1/2, which we have shown previously to cause phosphorylation of gIVA-PLA_2 in human eosinophils (17). Our 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 not for TNFα. Nevertheless, our data have not explored all possible inhibitory pathways. Specifically, we did not examined the potential role of p38 MAPK, since 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 also may be active in regulating β_2-integrin, e.g. phosphatidylinositide 3-kinase (PI3K). In prior studies, G-protein-mediated adhesion has been shown to be regulated by ERK-1/2-gIVA-PLA_2-interaction (18, 30). In this study, we showed a definite relationship 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, our data demonstrate the ability to block integrin adhesion is stimulus-dependent and directly corresponds to ERK-1 mediated gIVA-PLA_2 phosphorylation. We note especially that we cannot predict the therapeutic efficacy of PDE4 inhibition of PMN adhesion and transendothelial migration from these studies. However, TNFα-induced resistance to downregulation of adhesion caused by cAMP mediated mechanisms indicates that the circumstances of activation may, in large part, determine the efficacy of anti-neutrophilic therapies.

In summary, we find that TNFα prevents cAMP-induced inhibition of ERK-1 phosphorylation, which further prevents blockade of β_2-integrin adhesion. By contrast, LTB_4-activated PMNs with comparable increase in cAMP elicited by cilomilast demonstrated both inhibition of ERK-1 and gIVA-PLA_2 phosphorylation and blockade of β_2-integrin adhesion. The mechanism by which gIVA-PLA_2 causes β_2-integrin adhesion remains undefined. Accordingly, while we have identified that TNFα activated cells are refractory to cAMP-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 PMN depends critically upon the mode of activation.

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Figure Legends

Figure 1. Effect of mAb directed against β_1/β_2-integrins on FMLP-stimulated neutrophil adhesion to BSA or ICAM-1 coated microplate wells. PMNs were preincubated 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 *Materials and Methods section*. Each point represents the mean ± SEM.

Figure 2. Quantitation of PDE4 activity. Isolated human PMNs (PMN)s or eosinophils (EOS) were treated with buffer alone or 10^{-6} M cilomilast (CLM), and PDE4 activity was measured as described in *Materials and Methods.* PDE4 activity was expressed as fmol/min/10^6 cells and data are mean ± SEM for three independent experiments. *$P < 0.001$ for unstimulated PMN versus EOS.

Figure 3. Kinetics and concentration–dependent effect of LTB_4 and TNFα on adhesion. PMNs were activated with increasing concentrations of (A) LTB_4 for 15 min (*$p < 0.01$ for $>10^{-9}$ M LTB_4 vs control) or (B) TNFα for 30 min prior to adhesion assay (*$p < 0.01$ for > 10 ng/ml TNFα vs control). (C) Time course for adhesion for TNFα- or LTB_4-activated PMNs. Untreated PMNs were added to BSA-coated wells and activated with optimal concentration of LTB_4 (10^{-7} M) or TNFα (30 ng/ml) at various times, and adhesion was measured as a function of residual myeloperoxidase activity. Data are expressed as mean ± SEM for six independent experiments.

Figure 4. Effect of cilomilast and/or salmeterol on surface expression of CD11b surface expression. PMNs were preincubated with either 10^{-8} M to 10^{-6} M cilomilast alone (CLM; ■), 10^{-7} M salmeterol (SALM) alone, or cilomilast + 10^{-7} M salmeterol prior to activation with (A) 10^{-7} M LTB_4 for 15 min or (B) 30 ng/ml TNFα for 30 min at 37 °C. Surface CD11b expression was analyzed by flow cytometry and data are expressed as specific fluorescence intensity (SFI; mean fluoresecence intensity – unstimulated control). [*$p < 0.05$ for LTB_4-activated PMNs (no cilomilast, no salmeterol) versus 10^{-7} M cilomilast + LTB_4 with or without salmeterol. ** $p < 0.01$ for 10^{-6} M cilomilast + LTB_4 with or without salmeterol. p = NS for TNFα-activated PMNs vs all treatments and concentrations].

Figure 5. Effect of PDE4 inhibitors [cilomilast (CLM) or rolipram] and/or salmeterol (SALM) on stimulated adhesion. PMNs were preincubated with either a) cilomilast and/or salmeterol or b) rolipram and/or salmeterol prior to activation with 10^{-7} M LTB_4 (A/B) for 15 min or 30 ng/ml TNFα (C/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. [*$p < 0.05$, **$p< 0.01$ versus LTB_4-activated PMN (no cilomilast, no salmeterol). p = NS for TNFα-activated PMN (no cilomilast, no salmeterol) vs all concentrations of cilomilast or cilomilast + salmeterol].

Figure 6. Effect of cilomilast (CLM) and/or salmeterol (SALM) on intracellular cAMP concentration. PMNs were preincubated with either 10^{-10} M to 10^{-6} M cilomilast alone or cilomilast + 10^{-7} M salmeterol prior to activation with 10^{-7} M LTB_4 for 15 min or 30 ng/ml TNFα for 30 min at 37 °C and measurement of intracellular cAMP concentration. [*$p < 0.01$ for 10^{-6} M

cilomilast + LTB_4-activated PMNs versus 10^{-6} M cilomilast alone treated PMNs, **$p < 0.01$ for PMNs treated with 10^{-6} M cilomilast + 10^{-7} M salmeterol + LTB_4 versus 10^{-6} M cilomilast + LTB_4, no salmeterol, $^{\dagger}p < 0.01$ for PMNs treated with 10^{-6} M cilomilast + 10^{-7} M salmeterol + TNFα versus 10^{-6} M cilomilast + TNFα, no salmeterol]. Data (n = four) are mean ± SEM and expressed as picomole per 2.5 $x10^5$ cells (pmol/2.5 $x10^5$ cells).

Figure 7. Inhibitory effect of PDE4 inhibitors [cilomilast (CLM) or rolipram] and/or salmeterol (SALM) on ERK-1/2 phosphorylation. A representative ERK-1/2 phosphorylation caused by LTB_4 or TNFα-stimulated PMNs in the presence of absence of 10^{-6} M cilomilast (A) or rolipram (B) and/or 10^{-7} M salmeterol. Treated PMNs were lysed and loaded onto 10% SDS-PAGE, followed by immunoblotting analysis using anti-phosphorylation specific ERK-1/2 Ab. Equal sample loading was confirmed by using total anti-ERK-1/2 Ab.

Figure 8. Effects of cilomilast and/or salmeterol on gIVA-PLA_2 and Raf-1 phosphorylation. A representative (A) gIVA-PLA_2 phosphorylation and (B) Raf-1 phosphorylation caused by LTB_4 or TNFα-stimulated PMNs in the presence of absence of 10^{-6} M cilomilast and/or 10^{-7} M salmeterol. Treated PMNs were lysed and loaded onto 10% SDS-PAGE, followed by immunoblotting analysis using anti-phosphorylation Ser^{505} specific gIVA-PLA_2 Ab (A) and anti-phosphorylation Ser^{259} Raf-1 Ab (B). Equal sample loading was confirmed by using total anti-gIVA-PLA_2 Ab.

Figure 1.

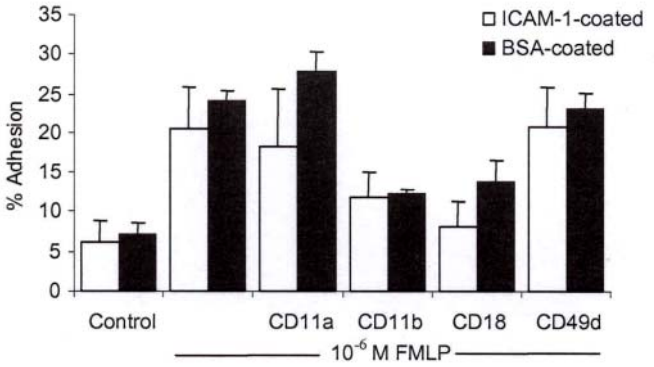

ICAM-1-coated
BSA-coated
% Adhesion
35
30
25
20
15
10
5
0
Control
CD11a
CD11b
CD18
CD49d
10-6 M FMLP

Figure 2

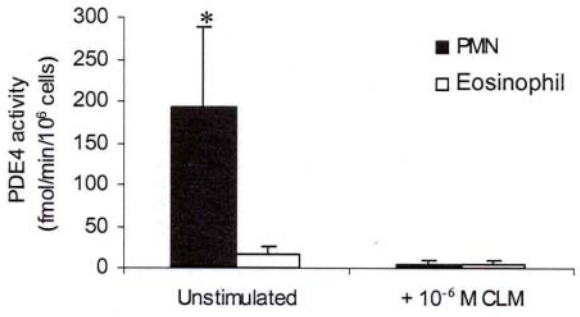

300
250
200
150
100
50
0
PDE4 activity
(fmol/min/10⁶ cells)
*
PMN
Eosinophil
Unstimulated
+ 10⁻⁶ M CLM

Figure 3

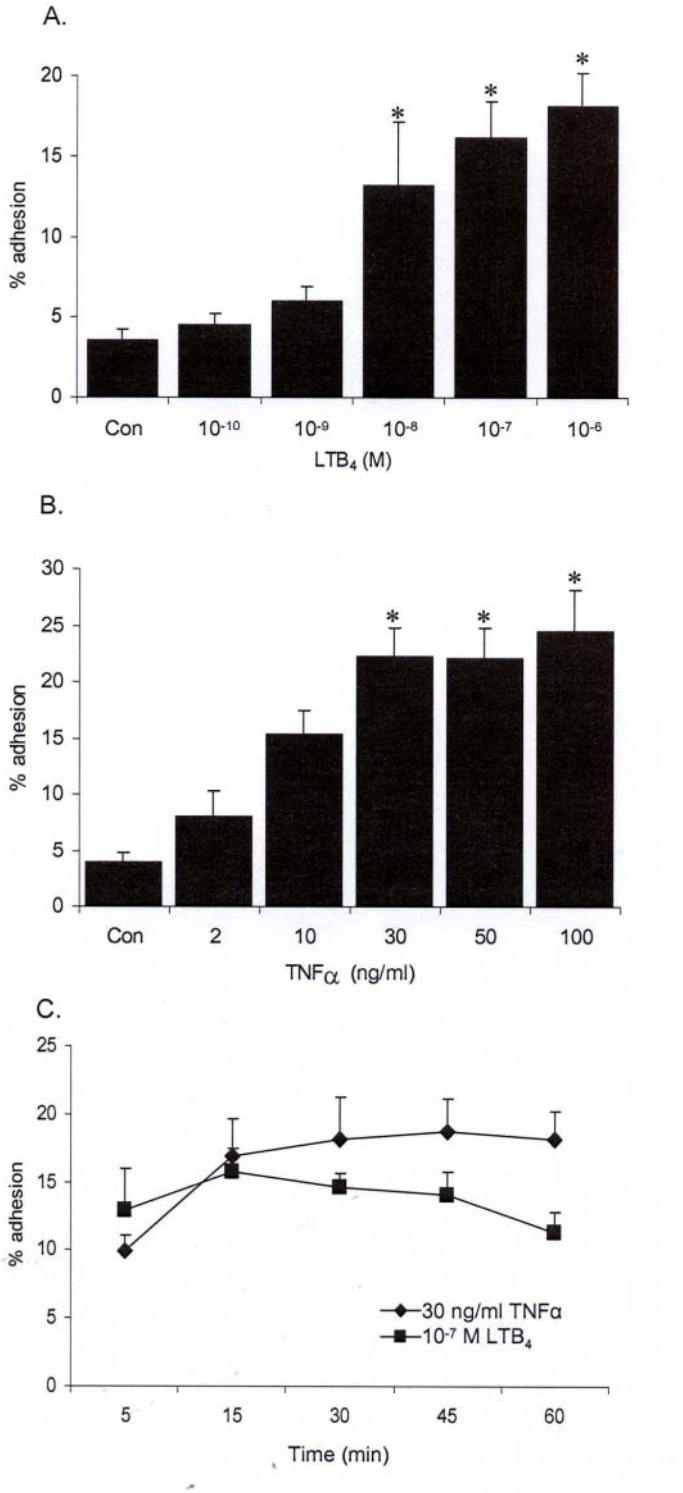

A.
% adhesion
20
15
10
5
0
Con
10-10
10-9
10-8
10-7
10-6
LTB4 (M)
B.
% adhesion
30
25
20
15
10
5
0
Con
2
10
30
50
100
TNFα (ng/ml)
C.
% adhesion
25
20
15
10
5
0
30 ng/ml TNFα
10-7 M LTB4
5
15
30
45
60
Time (min)

Figure 4

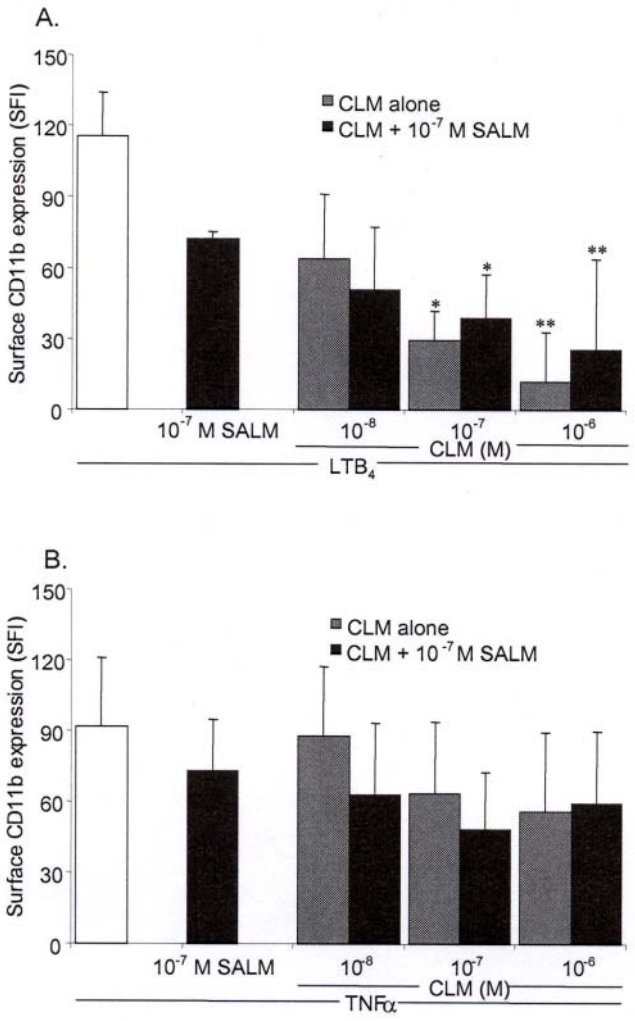

A.
Surface CD11b expression (SFI)
150
120
90
60
30
0
CLM alone
CLM + 10-7 M SALM
*
*
**
**
10-7 M SALM
10-8
10-7
10-6
CLM (M)
LTB4
B.
Surface CD11b expression (SFI)
150
120
90
60
30
0
CLM alone
CLM + 10-7 M SALM
10-7 M SALM
10-8
10-7
10-6
CLM (M)
TNFα

Figure 5.

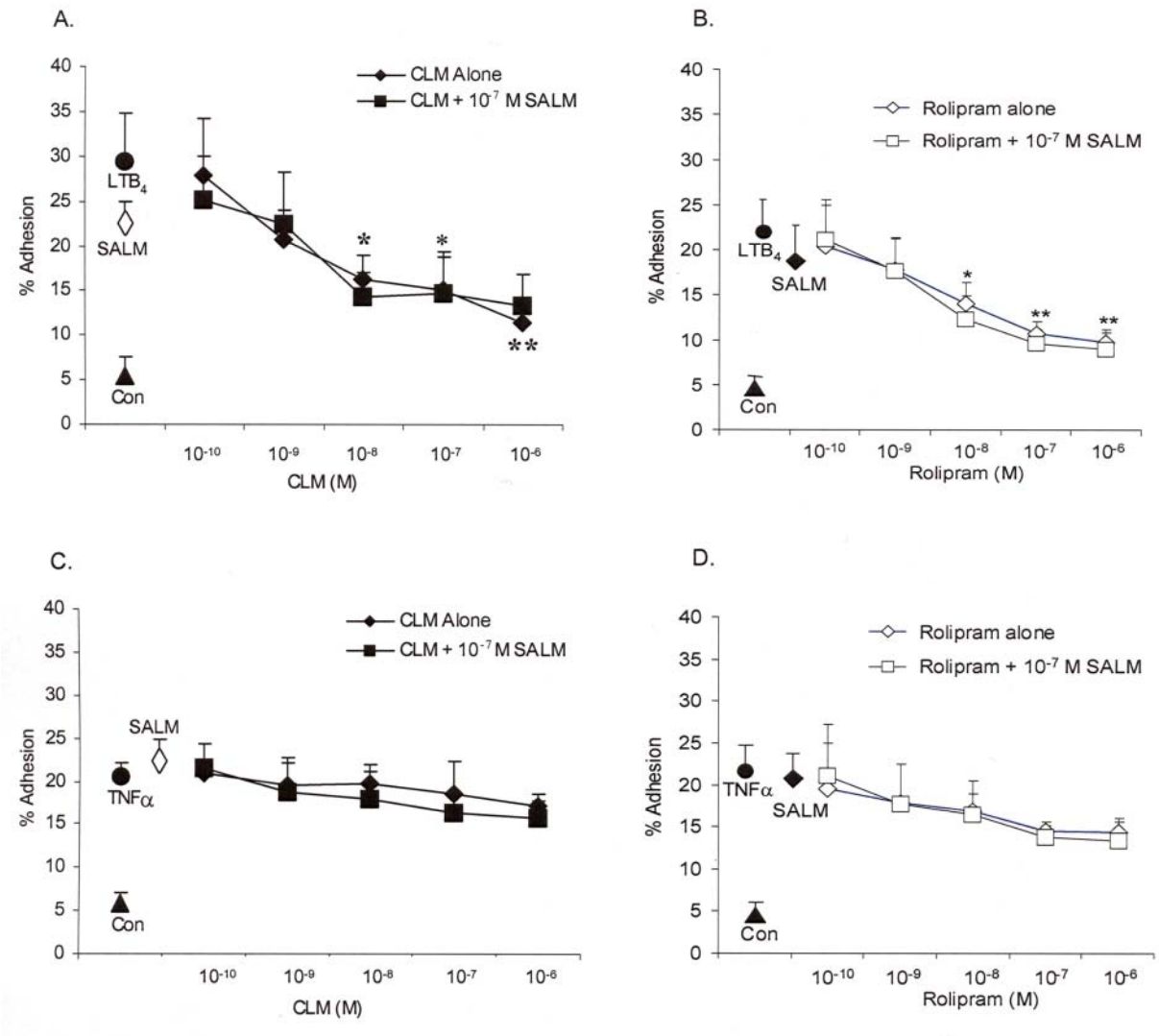

A.
CLM Alone
CLM + 10-7 M SALM
LTB4
SALM
Con
% Adhesion
CLM (M)
B.
Rolipram alone
Rolipram + 10-7 M SALM
LTB4
SALM
Con
% Adhesion
Rolipram (M)
C.
CLM Alone
CLM + 10-7 M SALM
SALM
TNFα
Con
% Adhesion
CLM (M)
D.
Rolipram alone
Rolipram + 10-7 M SALM
TNFα
SALM
Con
% Adhesion
Rolipram (M)

Figure 6

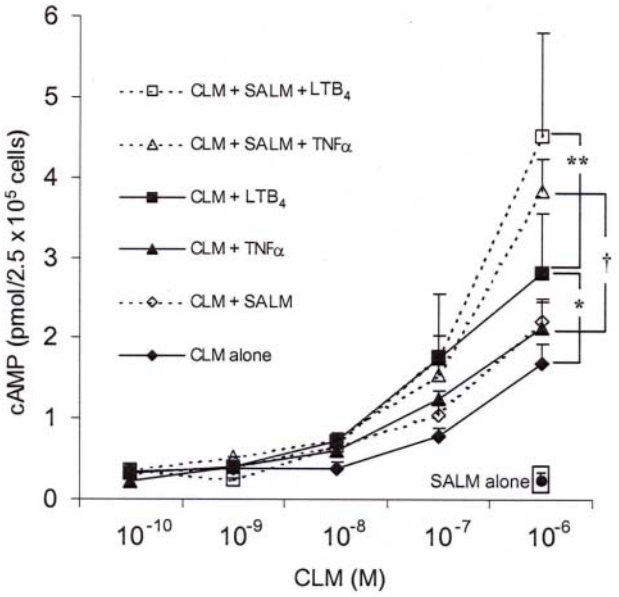

cAMP (pmol/2.5 x 10^5 cells)
CLM + SALM + LTB4
CLM + SALM + TNFα
CLM + LTB4
CLM + TNFα
CLM + SALM
CLM alone
SALM alone
**
*
†
10^-10
10^-9
10^-8
10^-7
10^-6
CLM (M)

Figure 7.

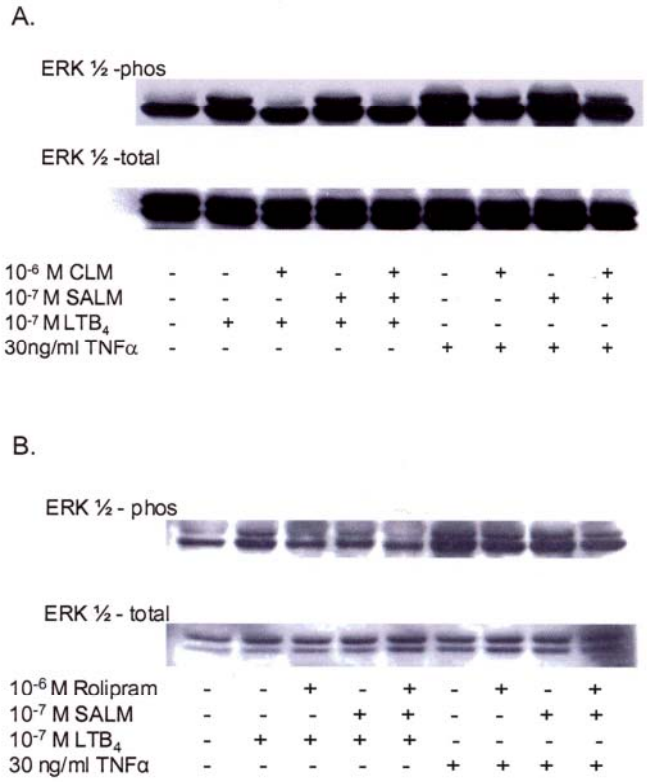
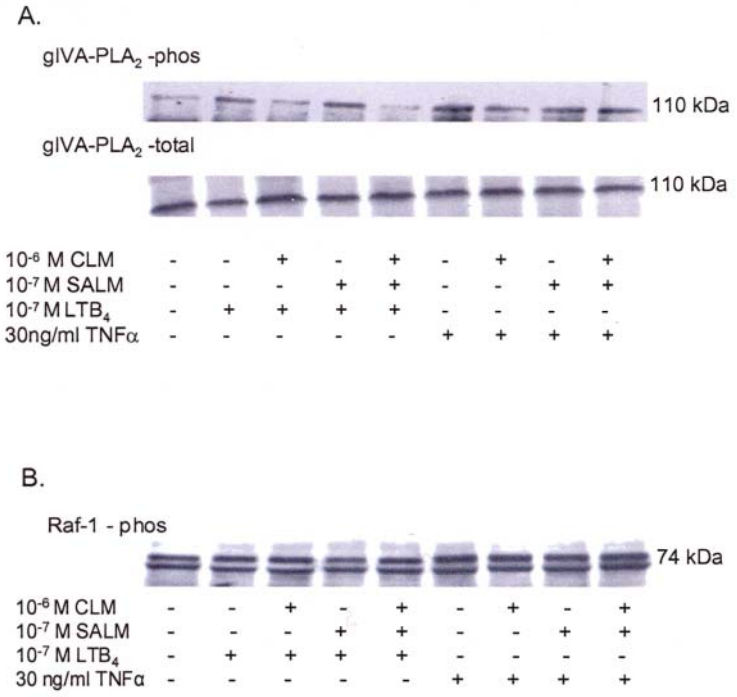

A.
ERK ½ -phos
ERK ½ -total
10-6 M CLM - - + - + - + - +
10-7 M SALM - - - + + - - + +
10-7 M LTB4 - + + + + - - - -
30ng/ml TNFα - - - - - + + + +
B.
ERK ½ - phos
ERK ½ - total
10-6 M Rolipram - - + - + - + - +
10-7 M SALM - - - + + - - + +
10-7 M LTB4 - + + + + - - - -
30 ng/ml TNFα - - - - - + + + +

Figure 8.


A.
gIVA-PLA2 -phos
110 kDa
gIVA-PLA2 -total
110 kDa
10-6 M CLM - - + - + - + - +
10-7 M SALM - - - + + - - + +
10-7 M LTB4 - + + + + - - - -
30ng/ml TNFα - - - - - + + + +
B.
Raf-1 - phos
74 kDa
10-6 M CLM - - + - + - + - +
10-7 M SALM - - - + + - - + +
10-7 M LTB4 - + + + + - - - -
30 ng/ml TNFα - - - - - + + + +