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Modulation of cell adhesion molecule expression and function on human lung microvascular endothelial cells by inhibition of phosphodiesterases 3 and 4

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1 Expression of cell adhesion molecules (CAM) on the lung microvascular endothelium is believed to play a key role in the recruitment of leukocytes in pulmonary inflammation. Moreover, regulation of CAM expression may be an important mechanism through which this inflammation may be controlled. Experimental evidence has suggested that combined phosphodiesterase (PDE) 3 and 4 inhibitors increase cyclic AMP levels within cells greater than inhibition of either isoenzyme alone. In the present study we assessed the effect of combinations of rolipram (PDE4 inhibitor), ORG 9935 (PDE3 inhibitor) and salbutamol (β-agonist) on CAM expression and neutrophil or eosinophil adhesion to human lung microvascular endothelial cells (HLMVEC).

2 Tumour necrosis factor-α (TNF-α)-induced intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin expression were measured on HLMVEC monolayers at 6 h by a specific ELISA technique in the presence of different combinations of medium, rolipram, ORG 9935 and salbutamol.

3 Rolipram in combination with salbutamol, but neither agent alone, inhibited TNF-α-induced E-selectin expression, whilst ICAM-1 and VCAM-1 expression were not affected. ORG 9935 had no significant effect on CAM expression alone. However, in combination with rolipram a syngergistic inhibition of VCAM-1 and E-selectin, but not ICAM-1, expression was observed. No further inhibition was seen in the additional presence of salbutamol.

4 Neutrophil adhesion to TNF-α-stimulated (6 h) HLMVEC was mainly E-selectin dependent in this model, as ENA_2 an anti-E-selectin monoclonal antibody (mAb) abrogated neutrophil adhesion. Eosinophil adhesion was E-selectin-, ICAM-1- and VCAM-1-dependent, as assessed by the inhibitory activity of ENA_2 and the ability of a mAb to the ICAM-1 ligand, CD18, and a mAb to the VCAM-1 ligand, VLA_4, to attenuate adhesion.

5 Rolipram in the presence of salbutamol or ORG 9935 significantly inhibited neutrophil adherence to TNF-α-stimulated HLMVEC. Eosinophil adherence to monolayers was inhibited only when HLMVEC were activated in the presence of rolipram and ORG 9935.

6 Collectively, the findings presented in this manuscript suggest that inhibition of PDE4 with appropriate activation of adenylate cyclase is sufficient to inhibit induction of E-selectin expression on HLMVEC to a level that has functional consequences for neutrophil adhesion. In contrast, combined inhibition of PDE3 and 4 isoenzymes is necessary to inhibit VCAM-1 and to have inhibitory effects on eosinophil adhesion to activated HLMVEC. Upregulation of ICAM-1 expression on HLMVEC does not appear to be modulated by PDE3 and 4 inhibition. These data may have implications for the use of selective PDE4 inhibitors in lung inflammation.

Keywords: PDE inhibitors; lung microvascular endothelium; TNF-α; E-selectin; vascular cell adhesion molecule-1; intercellular adhesion molecule-1; leukocyte adhesion

Introduction

During inflammation locally released cytokines such as tumour necrosis factor (TNF)-α induce the expression of cell adhesion molecules (CAM) on endothelial cells (Carlos & Harlan, 1994; Malik & Lo, 1996). These adhesion molecules include intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) which bind to leukocytes via their ligands; (i) lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) and macrophage antigen-1 (Mac-1; CD11b/CD18), (ii) L-selectin, sialyl-Lewis X and related carbohydrate structures; and (iii) very late antigen-4 (VLA_4; $\alpha_4\beta_1$), respectively (Carlos & Harlan, 1994; Malik & Lo, 1996). Evidence from both *in vitro* and *in vivo* studies suggest that regulation of CAM expression on endothelial cells plays a key role in controlling leukocyte adhesion and migration during inflammatory diseases of the lung including asthma, adult respiratory distress syndrome (ARDS) and sepsis (Redl *et al*., 1991; Gosset *et al*., 1995; Grau *et al*., 1996; Moss *et al*., 1996). In patients with allergic asthma, significant increases in endothelial CAM expression from bronchial mucosa biopsies have been observed compared to normal subjects (Gosset *et al*., 1995). This increase in CAM has been correlated with eosinophil and total leukocyte infiltrate measured in bronchoalveolar lavage fluid (BALF) from these patients. Pulmonary endothelial cells taken from patients with ARDS (Grau *et al*., 1996) also show significant increases in ICAM-1 and VCAM-1 expression compared to controls, and inhibition of E-selectin function with a monoclonal antibody has protective effects on lung function in experimental and clinical sepsis (Ridings *et al*., 1995; Friedman *et al*., 1996).

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Recent interest has focused on intracellular signalling mechanisms involved in the regulation of CAM expression as a tool for modulating inflammation. In a wide range of cells and tissues, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) has proved to be an important target; increasing levels of this second messenger within cells (to activate protein kinase A (PKA) which in turn, phosphorylates other substrates; reviewed by Giembycz & Raeburn, 1991) has been shown to have anti-inflammatory effects (reviewed by Teixeira *et al*., 1997). Increasing cyclic AMP levels within cells by pharmacological manipulation can be achieved in two ways; by increasing the rate of synthesis of cyclic AMP from ATP via activation of adenylate cyclase, or by decreasing the rate of breakdown by hydrolysis to inactive 5′-monophosphates (Torphy & Undem, 1991). Hydrolysis of cyclic AMP and cyclic GMP is carried out by a family of seven distinct phosphodiesterase (PDE) enzymes (Beavo *et al*., 1994). Selective inhibition of these isoenzymes appears to be the most effective mechanism for controlling cyclic AMP levels within cells.

The use of specific PDE inhibitors in *in vitro* and *in vivo* models of inflammation has demonstrated a variety of therapeutic anti-inflammatory effects (Dent *et al*., 1994; Teixeira *et al*., 1997). Studies on bronchial smooth muscle have shown that PDE3 and 4 isoenzyme inhibitors are effective broncho-relaxants and may be effective in relieving airway obstruction in asthmatics in combination with β-agonists such as salbutamol (Polson & Strada, 1996). In addition to their bronchodilator action, selective PDE4 inhibitors suppress pro-inflammatory functions of a range of cells. Amongst their actions are inhibition of inflammatory cell activation (neutrophils, monocytes, lymphocytes, mast cells and eosinophils; Torphy & Undem, 1991; Giembycz and Dent, 1992; Dent *et al*., 1994), reduction in endothelial cell permeability (Suttorp *et al*., 1993; 1996), suppression of transendothelial leukocyte migration (Lidington *et al*., 1996) and inhibition of inflammatory cell recruitment into sites of inflammation (reviewed by Teixeira *et al*., 1997). In some cells and tissues the use of PDE3 and 4 inhibitors in combination has been shown to be more effective at suppressing cell function than inhibition of PDE 3 or 4 isoenzymes alone. These effects include inhibition of pulmonary endothelial monolayer permeability, regulation of IL-2 release from T-lymphocytes and a more effective relaxation of tracheal smooth muscle (Shahid *et al*., 1991; Giembycz *et al*., 1996; Suttorp *et al*., 1996).

Endothelial cells have been shown to express the isoenzymes PDE2, 3 and 4 (Suttorp *et al*., 1993; 1996), PDE3 and 4 being the major cyclic AMP hydrolyzing enzymes. By raising intracellular cyclic AMP levels with a non-selective PDE inhibitor (IBMX), TNFα-induced VCAM-1 and E-selectin expression on human umbilical vein endothelial cells (HUVEC) was partly inhibited (Pober *et al*., 1993; Ghersa *et al*., 1994). Inhibition of PDE4 isoenzyme activity in HUVEC (used in combination with adenylate cyclase activators and protein kinase A activators), has also been shown to inhibit E-selectin but not VCAM-1 expression (Morandini *et al*., 1996). However, The effect of PDE3 inhibition alone and in combination with PDE4 inhibition on TNF-α-induced CAM expression on endothelial cells has not been studied.

Therefore, the aim of the present study has been to investigate the effects of combined PDE3 and PDE4 inhibition with Org 9935 and rolipram, respectively, in the absence and presence of a β-agonist (salbutamol) on CAM expression and leukocyte adhesion to human lung microvascular endothelial cells (HLMVEC). With the recent isolation of human pulmonary microvascular endothelial cells (Carley *et al*., 1992), we have been able to use this *in vitro* model to investigate various aspects of pulmonary microvascular endothelial cell function. This may be useful as the basis for studies of adhesion molecule targeted therapies of pulmonary inflammatory disease.

Methods

Antibodies

Affinity isolated goat anti-(mouse immunoglobulins) gamma and light chain specific peroxidase conjugate was obtained from TCS Biologicals Ltd. Mouse anti-human ICAM-1 (mAb RR1/1) (Rothlein *et al*., 1988) was provided by Dr R. Rothlein (Boehringer Inglheim Pharmaceuticals, Ridgefield, CT, U.S.A.). Monoclonal antibodies BBIG-E1 (anti-E-selectin; Pigott *et al*., 1991), BBIG-V1 (anti-VCAM-1) and 2B4 (anti-VLA-4; Needham *et al*., 1994) were generous gifts from Dr R. Pigott (British Biotech, Oxford). $F(ab')_2$ fragments of the anti-CD18 mAb 6.5E (Andrew *et al*., 1993) and the anti-E-selectin mAb ENA_2 (Leeuwenberg *et al*., 1990) together with the control myeloma IgG_1 MOPC21 were generous gifts from Dr M. Robinson (Celltech, Slough).

Cell culture

HLMVEC used in this study have been shown to retain a number of properties of EC including the production of human Factor-VIII-related antigen, uptake of acetylated LDL and expression of CD31 (Shen *et al*., 1995). We have also confirmed that these cells were positive for Factor-VIII-related antigen and CD31 expression (data not shown). HLMVEC were maintained in EGM-MV medium, a modification of MCDB 131, supplemented with 10 ng ml^{-1} human recombinant epidermal growth factor, 1 μg ml^{-1} hydrocortisone, 5% heated-inactivated foetal calf serum (FCS), 50 μg ml^{-1} gentamicin, 50 ng ml^{-1} amphotericin-B, bovine brain extract containing 12 μg ml^{-1} protein, and 10 μg ml^{-1} heparin. Cells used between passage $5^{\circ}-10^{\circ}$ did not alter in terms of basal or TNF-α-induced CAM expression, and hydrocortisone in the culture medium also had no detectable effect on CAM expression (basal or TNF-α-stimulated). Confluent cells were washed with HBSS, trypsinized with 0.025% trypsin +0.01% EDTA and collected into trypsin neutralizing solution. Cells were seeded at a density of 3.2×10^3 cells per well onto 1% gelatin-coated flat-bottomed Nunclon 96-well microtitre plates.

Enzyme-linked immunosorbant assay (ELISA) for ICAM-1, VCAM-1 and E-selectin expression

ICAM-1, VCAM-1 and E-selectin were detected 4 days after seeding by an ELISA method (Pigott *et al*., 1991) by use of mouse anti-human ICAM-1 (RR1/1), VCAM-1 (BBIG-V1) or E-selectin (BBIG-E1) primary mAbs, and a peroxide-linked goat anti-mouse secondary antibody. Briefly, confluent HLMVEC in Nunclon 96 well plates, were incubated for 6 h with medium or TNF-α (0.1 or 1 ng ml^{-1}) in the absence or presence of combinations of rolipram (1–10 μM), salbutamol (10 μM) and ORG 9935 (1–10 μM). The vehicle, DMSO (0.1%) had no effect on basal ICAM-1 or TNF-α-induced CAM expression (data not shown). CAM expression was measured at 6 h as previous kinetic analysis demonstrated that

significant levels of ICAM-1, VCAM-1 and E-selectin were expressed at this time (Blease *et al.*, 1996). Following removal of stimuli, cells were washed three times with PBS containing Ca^{2+}, Mg^{2+} and 0.1% BSA and incubated for 45 min with 1 μg ml^{-1} RR1/1, BBIG-V1 or BBIG-E1. Primary antibody was removed by washing, and HLMVEC monolayers were incubated (45 min) with a 1 : 1000 dilution (in PBS + 10% goat serum) of goat anti-mouse peroxidase conjugate followed by incubation with ABTS, a peroxidase-sensitive substrate (1 mg ml^{-1}, in 0.2 M citrate/phosphate buffer, pH 5, containing 0.1% H_2O_2) for 30 min. The reaction was terminated by addition of 0.2 M citrate. All incubations were carried out at room temperature. Chromophore development was determined by measuring optical density (O.D.) at 405 nm (OD_{405}) by use of a Titretec MCC/340 Multiscan microplate reader. Background absorbance was determined from monolayers incubated without primary antibody and this value was then subtracted from the absorbance readings. The data presented are derived from O.D. readings which fall along the linear portion of the development curve.

Separation of human peripheral blood neutrophils or eosinophils

Granulocytes were isolated from peripheral blood of normal or mildly atopic adult donors for neutrophils or eosinophils respectively, by the method of Haslett *et al.* (1985). Briefly, blood was collected to a total volume of 40 ml into 3.8% citrate and spun for 20 min at 300 *g*. The platelet-rich plasma was removed, underlayed with 90% Percoll and spun at 2000 *g* for

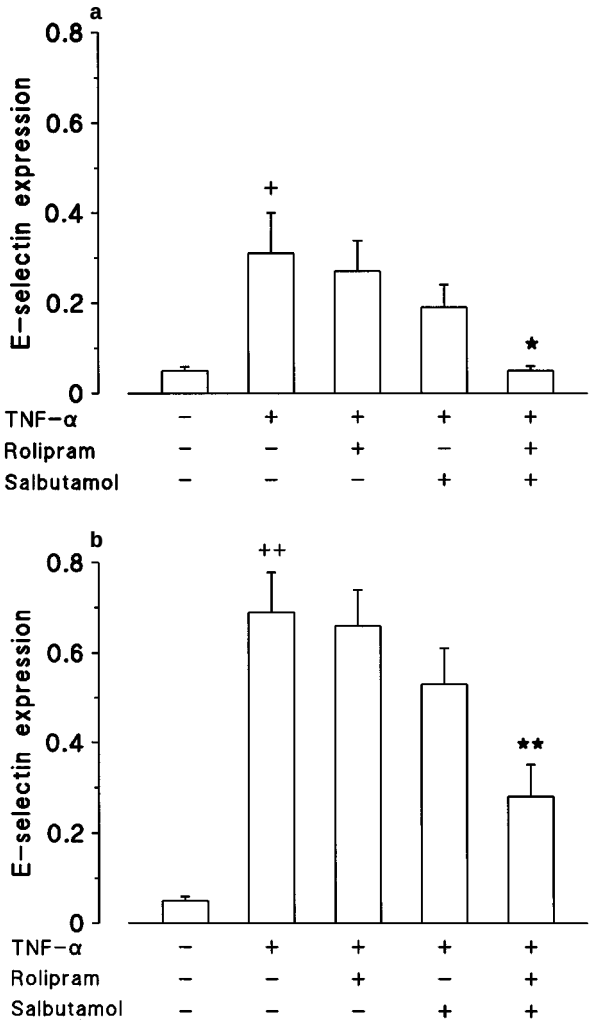

Figure 1 E-selectin expression on HLMVEC following 6 h exposure to (a) 0.1 ng ml^{-1} or (b) 1 ng ml^{-1} TNF-α in the absence and presence of rolipram (10 μM) and/or salbutamol (10 μM). Results are shown as means $\pm$ s.e.mean of 5 experiments. Statistical analysis was performed by use of one-way ANOVA followed by Dunnett's test which compares test values to a control (cell monolayers incubated with TNF-α). $^{+}P<0.01$ or $^{++}P<0.001$ show significant increases in E-selectin expression from control and $^{*}P<0.01$ or $^{**}P<0.001$ show significant decreases in E-selectin expression from levels induced by TNF-α.

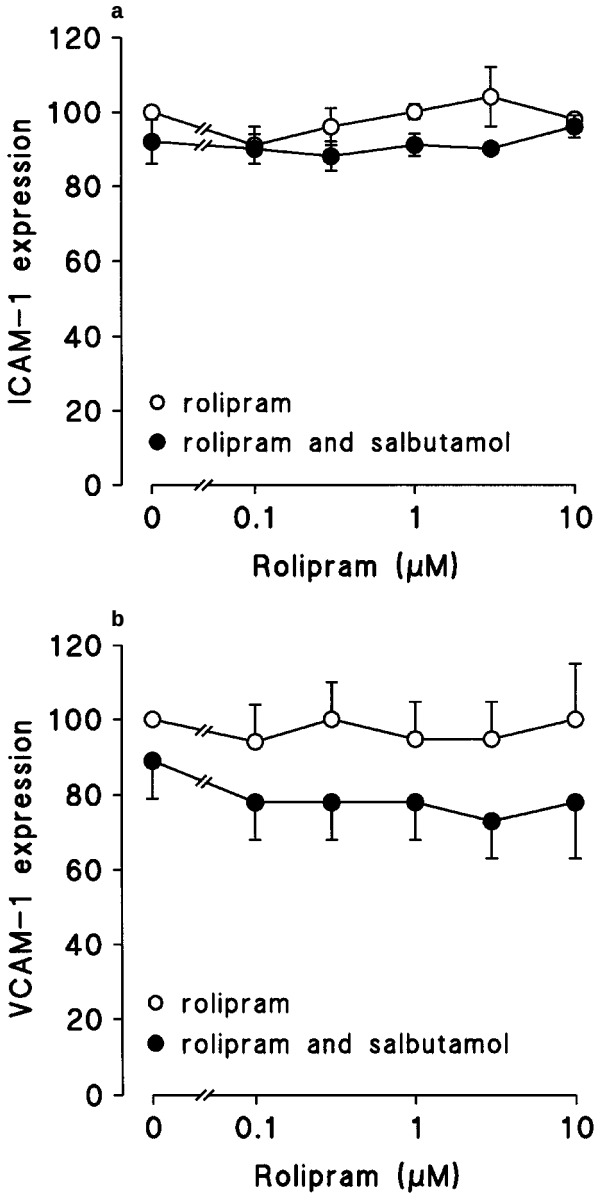

Figure 2 Effect of rolipram alone and in combination with salbutamol on TNFα-induced (a) ICAM-1 and (b) VCAM-1 expression on HLMVEC. Endothelial cells were stimulated for 6 h with TNF-α (1 ng ml^{-1}), with TNF-α and rolipram (0.1 – 10 μM) or with TNF-α, rolipram (0.1 – 10 μM) and salbutamol (10 μM). Results are expressed as % of TNF-α-induced CAM expression and are means of 5 experiments; vertical lines show s.e.mean. Basal and TNF-α induced ICAM-1 were (OD_{405}) 0.16 $\pm$ 0.03 and 0.72 $\pm$ 0.04, respectively while TNF-α-induced VCAM-1 expression was 0.29 $\pm$ 0.10. Statistical analysis was performed by use of one-way ANOVA followed by Dunnett's test which compares test values to a control (cell monolayers incubated with TNF-α).

20 min at room temperature (temperature used unless otherwise stated) to produce platelet-poor plasma (PPP). To the lower buffy coat produced by the first spin, 5 ml of 6% dextran was added and the volume made up to 50 ml with 0.9% saline. This was allowed to stand for 30 min for erythrocyte sedimentation to occur. The leukocyte-rich supernatant was removed and centrifuged at 300 *g* for 8 min. The pellet was resuspended in PPP and layered onto freshly prepared discontinuous Percoll-plasma gradients (42 and 51% Percoll in PPP) and centrifuged for 10 min at 260 *g*. The granulocyte band was collected, washed in PPP and resuspended in KRPD buffer (4.8 mM KCl, 3.1 mM NaH_2PO_4, 12.5 mM Na_2HPO_4, 5% glucose and 2% FCS). Granulocytes obtained from normal donors were >98% neutrophils. Granulocytes obtained from mildly atopic donors were used to purify eosinophils and were incubated with anti-CD16 microbeads to remove neutrophils (1/10 dilution when 40 l of beads were added per 10^8 granulocytes for 45 min). A Type-CS MACS separation column was set up in a magnetic separator and microbead-labelled granulocytes applied to the top of the column. Eosinophils (>97% pure) were eluted in 35 ml KRPD buffer. Eosinophils or neutrophils (5×10^6 cells ml^{-1}) were labelled (30 min, 37°C) with a fluorescent dye, calcein-AM (10 μM dissolved in 1% DMSO in KRPD without FCS). Cells were washed twice in KRPD without FCS and resuspended at 1.25×10^6 cells ml^{-1} in KRPD containing 2.5% FCS, 0.93 mM $CaCl_2$ and 1.2 mM $MgSO_4$ for the adhesion assay.

Measurement of neutrophil or eosinophil adhesion to lung microvascular endothelial cells

HLMVEC monolayers grown on 96-well plates were pretreated with TNF-α (1 ng ml^{-1}) and/or combinations of PDE inhibitors with or without salbutamol for 6 h. Monolayers were washed 3× with PBS (containing Ca^{2+}/Mg^{2+}) to remove stimuli before carrying out the adhesion assay. One hundred microlitres of MOPC21, 6.5E, ENA_2 or KRPD with Ca^{2+}/Mg^{2+} were added per well, followed by 100 μl of calcein-AM labelled neutrophils or eosinophils and the plate was incubated at 37°C for 30 min. Fluorescence was measured with a Biolite F1 plate reader (excitation wavelength of 485 25nm and emission wavelength of 530 25nm) before and after washing the plate twice with PBS containing 1% horse serum to remove non-adherent cells. % adhesion was expressed as fluorescence after washing the plate minus the background fluorescence, divided by fluorescence before washing plate minus background ×100.

Cell culture reagents

HLMVEC, prepared by Clonetics, (California, U.S.A.), were obtained as cryopreserved third passage (3) cultures from TCS Biologicals Ltd (Buckingham). Microvascular endothelial growth medium (EGM-MV), 0.025% trypsin + 0.01% EDTA, HBSS, trypsin neutralizing solution were also obtained from TCS Biologicals Ltd. Dulbecco's PBS (±Ca^{2+}/Mg^{2+}) was purchased from Gibco Laboratories (Paisley, Scotland).

Cytokines and other reagents

Human recombinant (hr) TNF-α was obtained from Boehringer Mannheim (Lewes, East Sussex; specific activity $>1 \times 10^8$ u mg^{-1}). The following products were purchased from Sigma Chemical Company Ltd. (Poole, Dorset): 2,2′-azinobis-(3-ethylbenthiazoline-6-sulfonic acid; ABTS), gelatin, goat serum, horse serum, foetal calf serum (FCS), hydrogen peroxide (H_2O_2) and dimethylsulphoxide (DMSO). Percoll and dextran were obtained from Pharmacia Biotech Ltd. (St. Albans), sterile normal saline (0.9%) from FL (Manufacturing) Ltd., Fresenius Health Care Group (Basingstoke) and very low endotoxin BSA from Bayer Ltd (Basingstoke). MACS CS separation columns and CD16 microbeads were purchased from Miltenyi Biotech (Camberley, Surrey). Salts for Krebs Ringer phosphate dextrose (KRPD) buffer were obtained from Sigma and BDH. Calcein-AM was obtained from Cambridge Bioscience (Cambridge). Rolipram was a gift from Dr N. Cooper, Chiroscience (Cambridge) and Org 9935 (4,5 - dihydro - 6-(5,6 -dimethoxy-benzo[b]thien-2-yl)-5-methyl-1(2H)-pyridazinone) was a gift from Dr Shahid, Organon Laboratories (Scotland).

Statistics

Results are expressed as mean±s.e.mean of *n* experiments. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the Dunnett's test or Bonferroni selected comparison test as indicated in figure legends. Results were considered significant if $P<0.05$.

Results

Effect of rolipram and salbutamol on TNF-β induced CAM expression

Stimulation of HLMVEC with TNF-α (0.1 and 1 ng ml^{-1}) for 6 h caused a significant increase in E-selectin expression (Figure 1a and b, respectively). In the presence of rolipram (10 μM) or salbutamol (10 μM), responses to both concentrations of TNF-α were not significantly altered. However, a combination of rolipram with salbutamol either abolished or caused a 64% inhibition of E-selectin expression induced by

Table 1 Effect of rolipram in the presence and absence of salbutamol on TNF-α–induced ICAM-1 and VCAM-1 expression on HLMVEC

	ICAM-1-expression		*VCAM-1-expression*	
Rolipram (μM)	*−Salbutamol*	*+Salbutamol*	*−Salbutamol*	*+Salbutamol*
0	100	95±6	100	106±8
0.1	87±4	81±9	93±7	96±8
0.3	85±8	78±4	91±7	90±4
1	95±3	83±4	90±3	96±1
3	97±11	84±3	86±8	93±4
10	98±1	97±9	83±2	85±1

HLMVEC were incubated for 6 h with TNF-α (0.1 ng ml^{-1}) with or without the addition of rolipram and salbutamol (10 μM). ICAM-1 and VCAM-1 expression are expressed as a percentage of responses to TNF-α-alone; basal ICAM-1 was (OD_{405}) 0.16±0.03, TNF-α-induced ICAM-1 was 0.32±0.10 and TNF-α-induced VCAM-1 was 0.15±0.05. Values are mean±s.e.mean of 4 experiments.

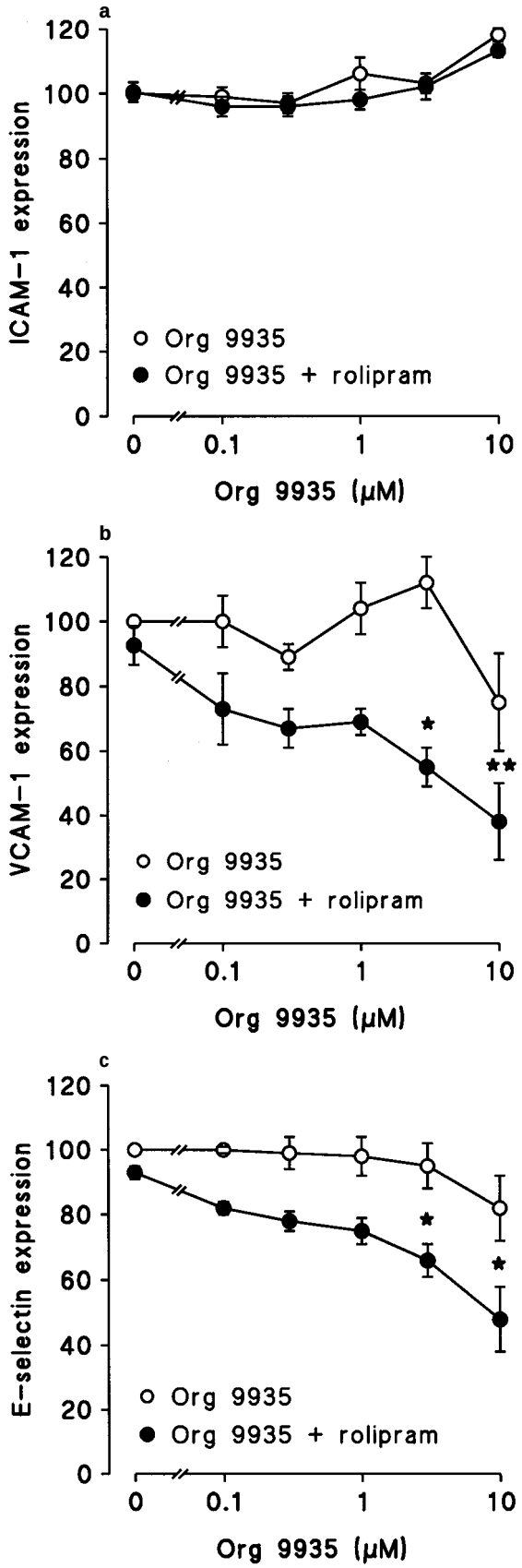

Figure 3 Effect of Org 9935 alone and in combination with rolipram on TNF-α-induced (a) ICAM-1, (b) VCAM-1 and (c) E-selectin expression on HLMVEC. Endothelial cells were stimulated for 6 h with TNF-α (1 ng ml^{-1}), with TNF-α and Org 9935 (0.1 – 10 μM) or with TNF-α, Org 9935 (0.1 – 10 μM) and rolipram (10 μM). Results are expressed as % of TNF-α-induced CAM expression and are means of 5 experiments; vertical lines show s.e.mean. Basal and TNF-α induced ICAM-1 were (OD_{405}) 0.15 ± 0.01 and 0.70 ± 0.01, 0.1 and 1 ng ml^{-1} TNF-α, respectively (Figure 1a, b). Concentration-response curves established that 10 μM rolipram provided the maximal inhibitory effect (data not shown).

Basal ICAM-1 expression (OD_{405} 0.16 ± 0.03) was not altered after 6 h incubation with rolipram (0.1 – 10 μM) alone or in combination with salbutamol (10 μM) (data not shown). In addition, TNF-α (1 ng ml^{-1}) induced ICAM-1 (0.72 ± 0.04) or VCAM-1 (0.29 ± 0.10) expression was not affected by rolipram alone or rolipram plus salbutamol (Figure 2a, b). Responses to the lower concentrations of TNF-α (0.1 ng ml^{-1}) were also unaffected by these compounds alone or in combination (Table 1).

Effect of Org 9935 alone and in combination with rolipram on TNF-α-induced CAM expression

The PDE3 inhibitor, Org 9935 (0.1 – 10 μM), alone or in combination with rolipram (10 μM), had no effect on basal (0.15 ± 0.01) or TNF-α-(1 ng ml^{-1}; 0.70 ± 0.04) induced ICAM-1 expression (Figure 3a). However, a combination of Org 9935 (3 and 10 μM) and rolipram (10 μM) caused a synergistic inhibition of TNF-α-(1 ng ml^{-1}) induced VCAM-1 and E-selectin expression (Figure 3b and c). Addition of salbutamol (10 μM) in the presence of Org 9935 (10 μM) did not alter the effect of Org 9935 alone, and salbutamol did not further reduce the inhibitory effect of Org 9935 plus rolipram on TNF-α-induced E-selectin or VCAM-1 expression (data not shown).

Contribution of CAM expression to neutrophil and eosinophil adhesion to HLMVEC

Before the functional consequences of PDE inhibition on TNFα-induced CAM expression were determined, the CAM requirement for adhesion of unstimulated neutrophils or eosinophils to HLMVEC monolayers was assessed by use of function-blocking mAbs.

Basal adhesion of neutrophils to untreated HLMVEC was significantly ($P<0.01$) increased following stimulation of HLMVEC for 6 h with TNF-α (1 ng ml^{-1}; Figure 4a). Compared to the control antibody (MOPC21, 10 μg ml^{-1}), the anti-E-selectin mAb (ENA_2, 10 μg ml^{-1}) reduced the adhesion of neutrophils to the level seen on untreated HLMVEC (Figure 4a). In contrast, the anti-CD18 mAb (6.5E, 10 μg ml^{-1}; a concentration that has been shown to be sufficient to block adhesion, see below) had no effect on neutrophil adhesion. These data suggest that under the conditions of the assay, the adhesion of unstimulated neutrophils which is increased as a result of TNF-α-activation of HLMVEC is solely due to E-selectin and that CD18/ICAM-1 interactions were not involved.

Eosinophil adhesion to TNF-α stimulated HLMVEC was significantly inhibited by the anti-CD18 mAb, 6.5E (10 μg ml^{-1}; $P<0.05$) and the anti-VLA_4 mAb, 2B4 (10 μg ml^{-1}; $P<0.05$). A combination of these two mAbs further reduced the adhesion of eosinophils to the level approaching that seen on unstimulated HLMVEC ($P<0.01$; Figure 4b). The anti-E-selectin mAb (ENA_2, 10 μg ml^{-1}) also

respectively, while TNF-α-induced VCAM-1 and E-selectin expression were 0.23 ± 0.03 and 0.61 ± 0.10, respectively. Statistical analysis was performed by use of one-way ANOVA followed by Dunnett's test which compares test values to a control (cell monolayers incubated with TNF-α). *$P<0.01$ or **$P<0.001$ denotes significant decreases in TNF-α induced CAM expression compared to control levels.

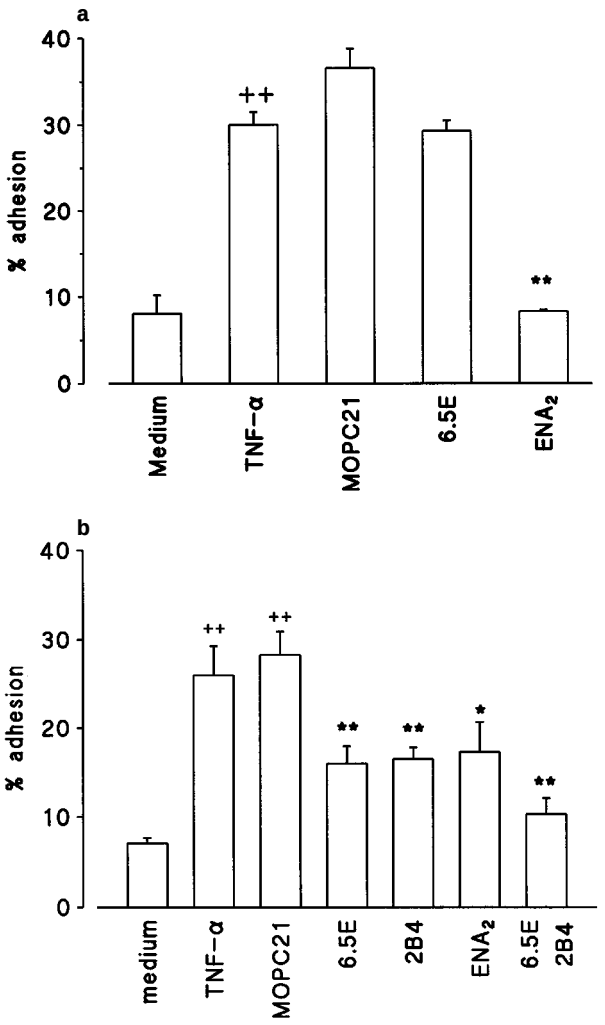

Figure 4 Adhesion of (a) neutrophils and (b) eosinophils to TNF-α stimulated HLMVEC and the effect of adhesion molecule mAbs. HLMVEC were stimulated with medium or TNF-α (1 ng ml^{-1}) for 6 h followed by addition of neutrophils or eosinophils and a control mAb MOPC21, anti-CD18 mAb 6.5E, anti-E-selectin mAb ENA_2, anti-VLA-4 mAb 2B4 or a combination of 6.5E and 2B4. All mAbs were used at a final concentration of 10 μg ml^{-1}. After 30 min, non-adherent granulocytes were removed by washing and the percentage adhesion calculated. Values are mean ± s.e.mean of 4 determinations in 2 experiments. Statistical analysis was performed by use of one-way ANOVA followed by Dunnett's test which compares test values to a control. $^{++}P<0.01$ denotes significant increases in adhesion compared to basal levels and $*P<0.05$ or $**P<0.01$, denotes significant decreases in adhesion compared to TNF-α induced levels.

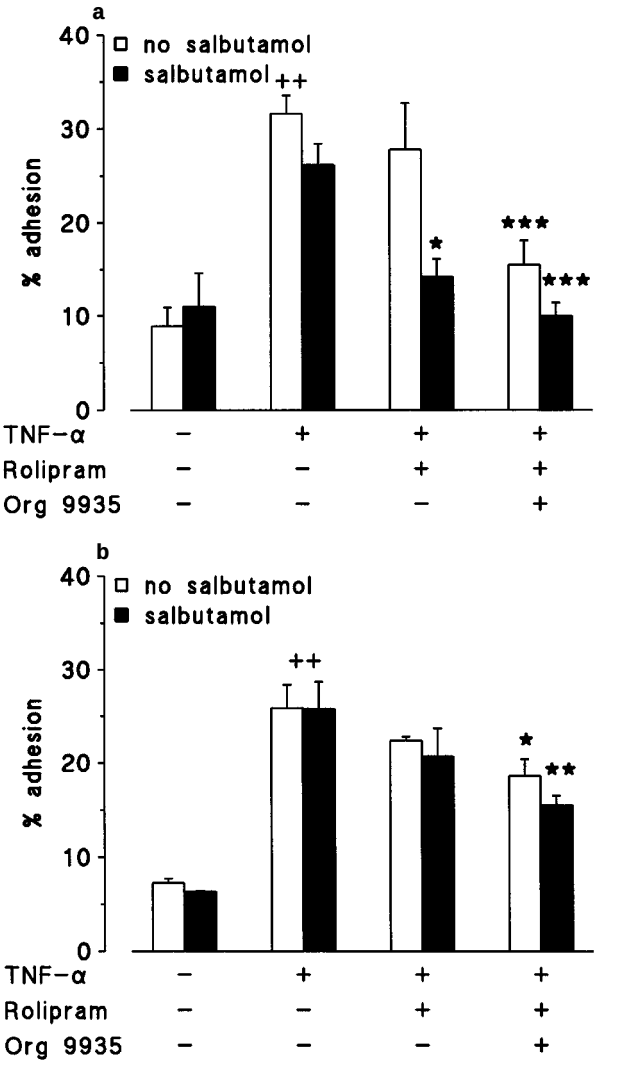

Figure 5 Effect of a combination of rolipram, Org 9935 and salbutamol on (a) neutrophil and (b) eosinophil adherence to HLMVEC monolayers. HLMVEC were stimulated for 6 h with medium or TNF-α (1 ng ml^{-1}) for 6 h in the absence or presence of rolipram (10 μM) or rolipram plus Org 9935 (10 μM). Incubations were also carried out in the presence of salbutamol (10 μM). After 30 min, non-adherent granulocytes were removed by washing and the percentage adhesion calculated. Values are mean ± s.e.mean of 3–6 determinations in 2–3 experiments. Statistical analysis was performed by use of one-way ANOVA followed by the Bonferroni selected comparison. $^{++}P<0.01$ denotes significant increases in adhesion compared to basal levels and $*P<0.05$ or $**P<0.01$, denotes significant decreases in adhesion compared to TNF-α induced levels.

had a significant effect when tested alone (Figure 4b) although it did not further reduce the inhibitory actions of 6.5E and 2B4. These data indicate that eosinophil adhesion was dependent on E-selectin, ICAM-1 and VCAM-1.

Effect of combined PDE3 and 4 inhibition on eosinophil and neutrophil adhesion to HLMVEC

Incubation of unstimulated HLMVEC with rolipram, salbutamol or Org 9935 alone or in combination, had no effect on basal adherence of neutrophils or eosinophils (data not shown). Adhesion of neutrophils to HLMVEC stimulated with TNF-α (1 ng ml^{-1}) in the presence of a combination of rolipram (10 μM) and salbutamol (10 μM) was significantly reduced compared with TNF-α alone ($P<0.05$; Figure 5a). A similar reduction was seen on HLMVEC stimulated in the presence of rolipram plus Org 9935 (10 μM) ($P<0.01$) and this was not further inhibited in a significant manner by the addition of salbutamol (Figure 5a). Inhibition of neutrophil adhesion correlates with changes in E-selectin expression in the presence of combinations of rolipram, Org 9935 or salbutamol as as shown in Figures 1b and 3c.

Eosinophil adhesion to TNF-α-treated HLMVEC in the presence of rolipram was not significantly reduced and addition of salbutamol had no further effect (Figure 5b). In contrast, activation of HLMVEC in the presence of rolpiram plus Org 9935 resulted in a significant suppression of eosinophil adhesion and this was not further inhibited, in a significant manner, by the addition of salbutamol (Figure 5b).

Discussion

Characterization and cellular distribution of cyclic phosphodiesterase isoenzymes together with the development of specific inhibitors has allowed the mechanisms and functional consequences of regulation of cyclic nucleotides within cells to be investigated. Within the lung (bovine tracheal smooth muscle, human bronchi and porcine pulmonary artery EC; Shahid *et al*., 1991; de Boer *et al*., 1992; Suttorp *et al*., 1993), PDE isoenzymes 1-5 have been identified. Of these isoenzymes, PDE3 and 4 are the major cyclic AMP hydrolyzing enzymes (Beavo *et al*., 1994). Evidence from *in vitro* and *in vivo* models of pulmonary inflammation have demonstrated that activation of endothelial cells to express CAM is a key step in the process of inflammation within the lung (Gosset *et al*., 1995; Ridings *et al*., 1995; Grau *et al*., 1996). Hence, identification of intracellular signalling mechanisms involved in CAM regulation on EC may allow the development of pharmacological agents to inhibit lung inflammation.

Modulation of cyclic AMP levels within endothelial cells has been shown to have beneficial anti-inflammatory effects, such as inhibition of EC permeability (Suttorp *et al*., 1996) and cell migration (Franzini *et al*., 1995). The majority of cyclic AMP hydrolyzing activity within endothelial cells has been attributed to PDE3 and 4 isoenzymes (Suttorp *et al*., 1993; 1996) and these enzymes are therefore key targets in order to increase endothelial cyclic AMP levels. The effects of combined PDE3 and 4 isoenzyme inhibition has been studied on endothelial permeability and cell migration, with beneficial anti-inflammatory effects being greater than individual isoenzyme inhibition (Suttorp *et al*., 1996). However, the effect of PDE3 and combined PDE3 and 4 inhibition on CAM expression on EC has not been studied, especially those derived from the human pulmonary microvasculature.

The present study demonstrated that inhibition of PDE4 isoenzymes with the selective inhibitor rolipram resulted in an attenuation of TNF-α-induced E-selectin expression on HLMVEC monolayers, but only when co-incubated with salbutamol (an adenylate cyclase activator). This suggests that cyclase activity is normally low such that inhibition of PDE4 by itself is insufficient to elevate intracellular cyclic AMP to levels that have functional consequences on CAM expression. No effects of rolipram in combination with salbutamol on TNF-α-induced ICAM-1 or VCAM-1 expression were observed. These results concur with previous work by Morandini *et al*. (1996) on HUVEC, where TNF-α-induced E-selectin (but not VCAM-1 or ICAM-1) expression was inhibited by a 5 fold higher concentration of rolipram, but only in combination with forskolin. Our data and that of Morandini *et al*. (1996) contrast with the observations of Pober *et al*. (1993) who found that elevation of intracellular cyclic AMP inhibited both E-selectin and VCAM-1 expression in response to TNF-α. However, in the latter study the non-specific PDE inhibitor isobutylmethylxanthine (IBMX) was used in combination with forskolin. Thus, the inhibitory effect on VCAM-1 expression could have been a consequence of multiple PDE inhibition, including PDE3 and 4. At variance with all this work is the study of Deisher *et al*. (1993), who were unable to alter TNF-α-induced E-selectin or VCAM-1 expression on HUVEC by use of the non-selective PDE inhibitor, pentoxyfilline or dibutyryl cyclic AMP. However, one explanation for the difference was that a combination of these agents was not tested and therefore the intracellular cyclic AMP concentrations may not have been elevated sufficiently.

In the present study combined PDE3 and 4 inhibition resulted in a synergistic inhibition of TNF-α-induced E-selectin and VCAM-1 expression, whilst no effect was seen on ICAM-1 expression. Thus, although rolipram and salbutamol failed to modify VCAM-1 significantly, rolipram plus Org 9935 was effective and addition of salbutamol did not produce further inhibition. In comparison, E-selectin expression was attenuated to a similar extent by either rolipram plus salbutamol or rolipram plus Org 9935. These observations suggest that E-selectin is more sensitive to changes in cyclic AMP levels than VCAM-1. Levels of cyclic AMP induced by combined PDE3 and 4 inhibition may be greater than those induced by PDE4 inhibition alone, reaching sufficient levels to inhibit VCAM-1 expression. That the combined effect of PDE3 and 4 inhibition results in higher levels of cyclic AMP is supported by work in the guinea-pig trachea and T lymphocytes, where this treatment resulted in a more effective inhibition of total cyclic AMP hydrolytic activity than either isoenzyme alone (Harris *et al*., 1989; Giembycz *et al*., 1996). The difference in sensitivity between E-selectin and VCAM-1 may be due to the presence of cyclic AMP response elements identified in mRNA E-selectin promoter regions (De Luca *et al*., 1994), the effect of cyclic AMP being at the level of transcription, as no effect of cyclic AMP on mRNA stability has been observed (Morandini *et al*., 1996). These results suggest that the variable effects on VCAM-1 expression observed by Morandini *et al*. (1996) by PDE4 inhibition alone may not be due to differences in culture conditions but rather to insufficient levels of intracellular cyclic AMP.

The effects of PDE inhibition on CAM expression were reflected in the functional assays. Activation of HLMVEC in the presence of rolipram plus salbutamol resulted in a significant decrease in neutrophil but not eosinophil adhesion. This effect correlated with a reduction in E-selectin expression and function, as the E-selectin mAb ENA_2 caused a significant inhibition of neutrophil but not eosinophil adhesion. Combined PDE3 and 4 inhibition also resulted in reduced neutrophil adhesion and this was not further reduced in the presence of salbutamol, again reflecting the E-selectin expression data. Eosinophil adhesion was inhibited when the HLMVEC were activated in the presence of rolipram and Org 9935. Eosinophils, unlike neutrophils, express the ligand for VCAM-1 (VLA_4; Walsh *et al*., 1991), and are therefore sensitive to changes in VCAM-1 expression in addition to alterations in E-selectin and ICAM-1. Eosinophils also utilized ICAM-1 to adhere, since adhesion was partly reduced by the anti-CD18 mAb. However, since ICAM-1 expression was not altered by PDE inhibition, the changes in eosinophil adhesion to HLMVEC in the presence of rolipram and Org 9935 are therefore considered to be mediated via suppression of both E-selectin and VCAM-1.

In conclusion, we have demonstrated that combined PDE3 and 4 isoenzyme inhibition results in a synergistic inhibition of TNF-α-induced E-selectin and VCAM-1 expression, which we speculate is mediated via increased levels of cyclic AMP. Inhibition of E-selectin expression resulted in a reduction of TNF-α-induced neutrophil adhesion, whilst eosinophil adhesion was reduced by inhibition of E-selectin and VCAM-1 expression. The results of this study suggest that combined PDE3 and 4 inhibition may result in a more effective inhibition of total cyclic AMP hydrolytic activity of lung microvascular endothelium than inhibition of either isoenzyme alone. This may have implications for the use of PDE4 inhibitors in lung inflammation.

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