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Rolipram inhibits leukocyte-endothelial cell interactions *in vivo* through P- and E-selectin downregulation

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1 Rolipram, a selective phosphodiesterase (PDE) type 4 inhibitor, was used to characterize leukocyte recruitment mechanisms in models of acute and subacute inflammation. Intravital microscopy within the rat mesenteric microcirculation was employed.

2 Mesentery superfusion with PAF (0.1 μM) induced a significant increase in leukocyte rolling flux, adhesion and emigration at 60 min. Rolipram pretreatment, markedly inhibited these parameters by 100, 95 and 95% respectively.

3 Similar effects were observed when the mesentery was superfused with LPS (1 μg ml^{-1}) for the same time period and these leukocyte parameters were nearly abrogated by rolipram pretreatment.

4 LPS exposure of the mesentery for 4 h caused a greater increase in leukocyte rolling flux, adhesion and emigration which were inhibited by rolipram administration by 51, 71 and 81% respectively.

5 Immunohistochemistry revealed a significant increase in P-selectin expression after 60 min superfusion with PAF which was attenuated by rolipram.

6 LPS exposure of the mesentery for 4 h caused a significant increase in P- and E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression. Rolipram pretreatment down-regulated both P- and E-selectin expression but had no effect on ICAM-1 and VCAM-1 expression.

7 Significant increases in plasma cyclic AMP levels were detected at 4.5 h after rolipram administration.

8 In conclusion, we have demonstrated that rolipram is a potent *in vivo* inhibitor of leukocyte-endothelial cell interactions. The effects observed are mediated through endothelial P- and E-selectin downregulation. Therefore, selective PDE-4 inhibitors may be useful in the control of different inflammatory disorders.

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Keywords: Rolipram; adhesion molecules; cyclic AMP; leukocyte; endothelium; intravital microscopy

Abbreviations: CAM, cell adhesion molecule; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; DMSO, dimethylsulphoxide; Dv, venular diameter; HLMVECs, human lung microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; mAb, monoclonal antibody; MABP, mean arterial blood pressure; PDE, phosphodiesterase; PKA, protein kinase A; TNF-α, tumour necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; V_{mean}, mean red blood cell velocity; V_{rbc}, centreline red blood cell velocity; V_{wbc}, leukocyte rolling velocity

Introduction

Recruitment of leukocytes from the blood compartment into tissues represents an essential defensive step for the host's immune and inflammatory responses. However, the deregulation of these protective mechanisms may exist in a wide range of chronic inflammatory conditions including prevalent pathologies such as asthma, chronic obstructive pulmonary disease, atopic dermatitis, rheumatoid arthritis, and various neurological disorders (Teixeira *et al*., 1997). The development of drugs with an effective anti-inflammatory profile, but with fewer side-effects than available steroids and nonsteroidal anti-inflammatory drugs, would be beneficial as there are few other effective treatments in diseases where an uncontrolled inflammatory response exists.

A strategy that has received much attention recently relates to the pharmacological control of the level of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in pro-inflammatory and immunocompetent cells. The intracellular level of cyclic nucleotides is closely regulated by at least eleven distinct families of phosphodiesterases (PDEs) which are enzymes responsible for the breakdown of cyclic nucleotides within cells (Nicholson *et al*., 1991; Giembycz, 2000). The cyclic AMP-specific PDE type 4 (PDE-4) is particularly

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abundant in inflammatory and immune cells where it constitutes the major if not the sole cyclic AMP metabolizing enzyme found in most of these cells. Since an increase in cyclic AMP negatively regulates the synthesis and release of pro-inflammatory mediators, cytokines and reactive oxygen species from inflammatory and immune cells, PDE-4 has become an important molecular target for development of new drugs displaying widespread anti-inflammatory and immunomodulatory activities (Souness *et al*., 2000; Doherty, 1999). Results of preclinical and clinical studies with selective PDE-4 inhibitors have shown that this new class of drugs is of great potential therapeutic utility in chronic inflammatory diseases (Torphy & Page, 2000; Barnette & Underwood, 2000; Giembycz, 2000).

An impressive property of PDE-4 inhibitors is their ability to suppress inflammatory cell infiltration in different *in vivo* animal models of inflammation whereas inhibitors of other PDE isoenzymes are generally without effect (Teixeira *et al*., 1997; Torphy, 1998). Recent interest has focused on the intracellular signalling mechanisms involved in the regulation of cell adhesion molecule (CAM) expression as a tool for modulating inflammation. In a wide range of cells and tissues, cyclic AMP has proved to be an important target. Increasing levels of this second messenger within the cells activates protein kinase A (PKA) which in turn, phosphorylates other substrates and has been shown to have anti-inflammatory effects (Giembycz & Raeburn, 1991; Teixeira *et al*., 1997). Indeed, evaluation of cyclic AMP inhibits the expression of several CAMs and a diverse range of leukocyte functions. In this way, rolipram, a selective PDE-4 inhibitor, can decrease leukocyte recruitment and function as well as cause downregulation of both leukocyte and endothelial CAMs expression (Santamaria *et al*., 1997; Teixeira *et al*., 1997; Blease *et al*., 1998).

Despite these findings, most of the studies carried out regarding the effect of cyclic AMP elevating agents on CAM expression have been performed using *in vitro* adhesion models, with variable results. Therefore, the present study was undertaken to evaluate which CAMs are effectively modulated by PDE-4 inhibition *in vivo* under short-term acute (1 h) and longer-term subacute (4 h) inflammatory conditions to investigate properly its effect on constitutive, preformed and inducible CAMs expression. Intravital microscopy within the rat mesenteric microcirculation was used to examine the effect of rolipram pretreatment on CAMs mediating leukocyte-endothelial cell interactions during acute inflammation induced by PAF and LPS, and subacute inflammation using an LPS-induced model. Finally, immunohistochemical studies of the vascular bed under investigation were carried out at the end of each experiment to clarify the adhesive mechanisms involved in rolipram inhibition of leukocyte recruitment.

Methods

Animal preparation

Male Sprague–Dawley rats (200–250 g) were fasted for 20–24 h prior to experiments with free access to water. The animals were anaesthetized with sodium pentobarbitone (65 $mg^{-1}\,kg^{-1}$, i.p.). A tracheotomy was performed to facilitate breathing and the right jugular vein was cannulated for intravenous administration of drugs or additional anaesthetic as required. The right carotid artery was cannulated to monitor systemic arterial blood pressure through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.).

Intravital microscopy

A midline abdominal incision was made and a segment of the mid-jejunal mesentery exteriorized and carefully placed on an optically clear viewing pedestal to allow transillumination of a 3 cm^2 segment of the mesenteric microvasculature. The temperature of the pedestal was maintained at 37°C. Animal temperature was monitored using a rectal electrothermometer and maintained at the same temperature with an infrared heat lamp. The exposed intestine was continuously superfused with a bicarbonate buffer saline (BBS, pH 7.4, 2 ml min^{-1}, 37°C) and covered with a BBS-soaked gauze to prevent evaporation. Mesenteric microcirculation was observed through an orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a 20× objective lens (Nikon SLDW) and a 10× eyepiece as previously described (Alvarez *et al*., 2001a). A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a colour monitor (Sony Trinitron PVM-14N2E) and the images were captured on videotape (Sony SVT-S3000P) with superimposed time and date for subsequent playback analysis. The final magnification of the image on the monitor was 1300×.

Single unbranched mesenteric venules with diameters ranging between 25 and 40 μm were studied. Venular diameter was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX, U.S.A.). Centreline red blood cell velocity (V_{rbc}) was also measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc}\ 1.6^{-1}$) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)\ s^{-1}$, in which D_v is venular diameter (House & Lipowsky, 1987).

The number of rolling, adherent and emigrated leukocytes was determined off-line during playback analysis of videotaped images. Rolling leukocyte flux was determined by counting the number of leukocytes rolling passing a fixed reference point in the microvessel per min. The same reference point was used throughout the experiment as leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined by measuring the time required for a leukocyte to traverse a distance of 100 μm along the length of the venule and was expressed as $\mu m\ s^{-1}$. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or exceeding 30 s. Adherent cells were expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field. The rate of emigration was

determined from the difference between the number of any interstitial leukocytes present at the beginning of the experiment and the number of cells present in the intestitium at the end of the experiment.

Experimental protocol To determine the effect of PDE-4 enzyme inhibition on leukocyte infiltration elicited by platelet activating factor (PAF) or lipopolysaccharide (LPS), the selective PDE-4 inhibitor rolipram (8 mg kg^{-1}, i.p.) was given 30 min prior to stimulus suffusion to have proper plasma levels of its active metabolite. Rolipram was dissolved in dimethylsulphoxide (DMSO) and diluted further in saline (final concentration 15%) as previously described (Teixeira *et al*., 1994). The same percentage of DMSO was given i.p. to the rolipram control group 30 min before stimuli superfusion. The doses administered were the same as those used in other *in vivo* studies (Teixeira *et al*., 1994; Elwood *et al*., 1995). After a 30 min stabilization period, baseline measurements (time 0) of mean arterial blood pressure (MABP), V_{rbc}, vessel diameter, shear rate, leukocyte rolling flux and velocity and leukocyte adhesion and emigration were made. The superfusion buffer was then supplemented either with PAF (0.1 μM) or with LPS (1 μg ml^{-1}) and recordings were performed for 5 min at 15 min intervals over a 60 min period and the aforementioned leukocyte and haemodynamic parameters measured. In a separate groups of experiments the effect of buffer superfusion on leukocyte responses was evaluated for the same time period.

In another set of experiments, animals were pretreated with rolipram or vehicle and 30 min later 5 ml of LPS (0.2 μg ml^{-1}) was i.p. injected. Leukocyte and haemodynamic parameters were evaluated 4 h after LPS administration. Similarly, a group of rats were i.p. injected with saline and responses determined 4 h later.

Immunohistochemistry

Immunohistochemistry was used to examine the expression of the endothelial cell adhesion molecules P- and E-selectin, ICAM-1 and VCAM-1. Once the experiment using intravital microscopy was completed, both the superior mesenteric artery and superior mesenteric vein were rapidly cannulated for perfusion and fixation of the mesenteric circuit. The mesenteric tissue was first washed free of blood by perfusion with Krebs-Henseleit buffer warmed to 37°C. Once the venous perfusate was free of red blood cells, perfusion with 4% paraformaldehyde in phosphate-buffered saline was initiated. The tissue was perfusion-fixed in this manner for 3–5 min. The portion superfused with buffer and PAF for 60 min or that exposed to saline and LPS for 4 h was then isolated and further fixed in 4% paraformaldehyde for 90 min at 4°C as previously described (Weyrich *et al*., 1993). After fixation, the tissue was dehydrated using graded acetone washes at 4°C, embedded in paraffin wax and 4 μm-thick sections were cut.

Immunohistochemical localization of P-selectin, E-selectin, ICAM-1 and VCAM-1 was accomplished using a modified avidin and biotin immunoperoxidase technique as previously described by Weyrich *et al*. (1993). Treatment with microwave was performed when necessary. Slides were immersed in antigen unmasking fluid, placed in a plastic coplin jar and heat to boil in a microwave oven for 3–5 min. This was refilled if necessary with deionized water and microwaved for 3–5 min. The jar was placed at room temperature for 15 min and rinsed in deionized water. Tissue sections were incubated with the anti-rat-P-selectin mAb (RP-2, 12.2 mg ml^{-1}), or with the anti-rat-E-selectin mAb (RME-1, 14.6 mg ml^{-1}), or with the anti-rat-ICAM-1 mAb (1A29, 5 mg ml^{-1}), or with the anti-rat-VCAM-1 mAb (5F10, 6.8 mg ml^{-1}), for 24 h at a dilution of 1 : 50. Control preparations consisted in the incubation with the isotype matched murine antibody MOPC 21 (IgG_1, 10 mg ml^{-1}) or UPC 10 (IgG_{2a}, 10 mg ml^{-1}) as primary antibodies for the same period of time at a 1 : 50 dilution. Positive staining was defined as a venule displaying brown reaction product on greater than 50% of the circumference of its endothelium.

Plasma cyclic AMP measurements

The procedure followed was similar to that described by Cheng *et al*. (1997). Briefly, rats were i.p. injected with rolipram vehicle and 30 min later with saline. Another two groups of animals were either pretreated with rolipram or with vehicle and 30 min later 5 ml of LPS (0.2 μg ml^{-1}) was i.p. injected. After 4 h of saline or LPS administration animals were anaesthetized with sodium pentobarbitone (65 mg kg^{-1}), 5 ml of blood was collected by cardiac puncture and placed in tubes containing 50 μl of 50 mg ml^{-1} disodium EDTA. The samples were kept on ice and centrifuged at $10{,}000 \times g$ for 15 min at 4°C. The plasma samples were stored at −20°C before assay. Each sample was thawed at 23°C, diluted 10 fold an assayed for cyclic AMP in duplicate by cyclic AMP enzymeimmunoassay system following manufacturers instructions.

Statistical analysis All data are expressed as mean ± s.e.m. The data within groups were compared using an analysis of variance (one-way-ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisons. A P value <0.05 was considered to be statistically significant.

Materials Pentobarbitone, PAF, LPS (*Escherichia coli* serotype 0127:B8), rolipram, MOPC 21, UPC 10, disodium EDTA and DMSO were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Antibodies anti-rat-P-selectin (RP-2), anti-rat-E-selectin (RME-1) and anti-rat-VCAM-1 (5F10) were acquired as previously stated (Walter *et al*., 1997a,b; Sanz *et al*., 1997). Anti-rat-ICAM-1 (1A29) was supplied by LabClinics S.A., Barcelona, Spain. Stuf-Mark antigen unmasking fluid was from Serotec, Spain. Biotrak cyclic AMP enzymeimmunoassay was from Amersham Pharmacia Biotech (Uppsala, Sweden).

Results

Figure 1 illustrates PAF-induced leukocyte responses. Leukocyte rolling flux, adhesion and emigration were significantly increased within 15 min of 0.1 μM PAF superfusion. After 60 min superfusion with PAF, increases in leukocyte rolling flux (82.2 ± 11.9 vs 21.2 ± 5.1 cells min^{-1}) and concomitant significant decreases in the leukocyte rolling velocity (80.3 ± 18.6 vs 157.1 ± 35.3 μm s^{-1}) were observed vs buffer (Figure 1). Similarly, at the same time point, PAF

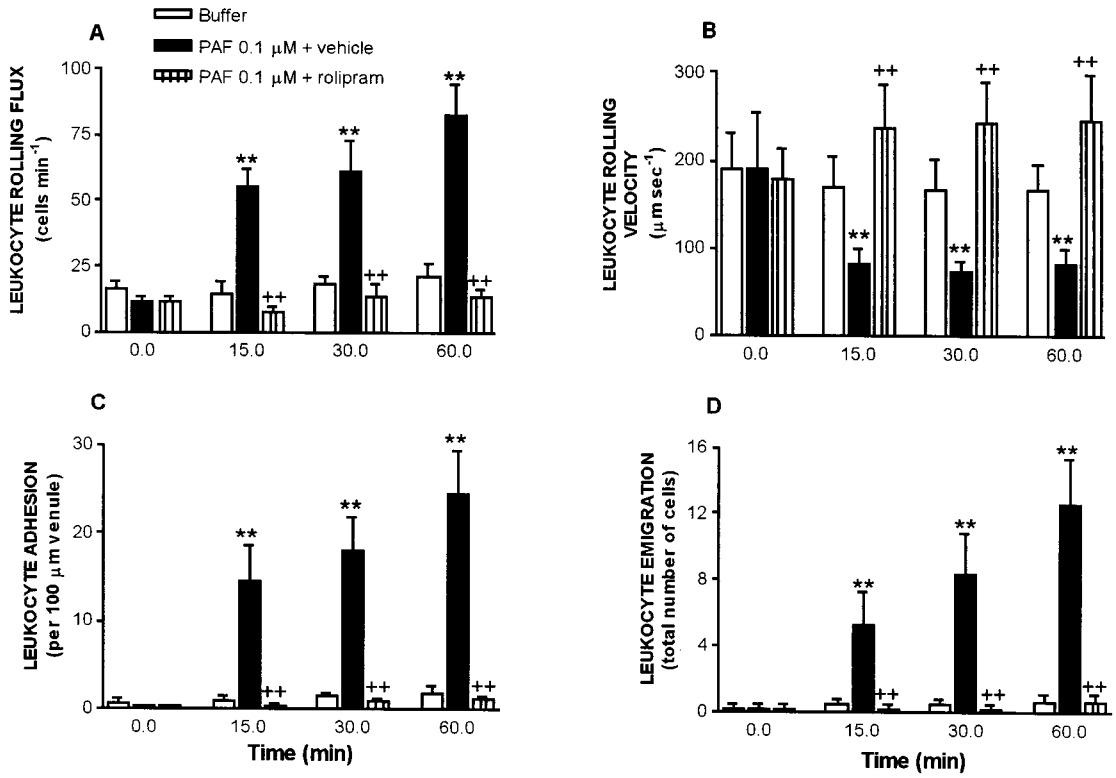

Figure 1 Effect of the PDE-4 inhibitor rolipram on PAF-induced leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C) and leukocyte emigration (D) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with buffer ($n=5$) or with PAF (0.1 μM) in animals untreated (rolipram vehicle, $n=5$) or pretreated with rolipram (8 mg kg^{-1} i.p., $n=5$). Results are represented as mean$\pm$s.e.mean. $^{**}P<0.01$ relative to values in the buffer group. $^{++}P<0.01$ relative to the PAF untreated group.

induced increases in leukocyte adhesion (24.4 ± 5.1 vs 1.8 ± 0.7 cells 100 μm^{-1}) and emigration (12.6 ± 2.7 vs 0.6 ± 0.4 cells field^{-1}) as shown in Figure 1. Pretreatment with rolipram markedly reduced the PAF-induced increase in leukocyte rolling flux, adhesion and emigration which were inhibited by 100, 95 and 95% respectively (Figure 1). In addition, the decrease in leukocyte rolling velocity induced by PAF at 60 min was reversed by the administration of rolipram (Figure 1).

Figure 2 presents the effect of rolipram on the leukocyte-endothelial cell interactions elicited by mesenteric exposure to 1 μg ml^{-1} LPS. In animals pretreated with rolipram vehicle, rapid leukocyte responses were also detected when LPS was superfused. Superfusion for 60 min with LPS vs buffer superfusion for the same time period, caused significant increases in leukocyte rolling flux (78.0 ± 19.1 vs 21.2 ± 5.1 cells min^{-1}), adhesion (12.8 ± 3.9 vs 1.8 ± 0.7 cells 100 μm^{-1}) and emigration (6.8 ± 1.3 vs 0.6 ± 0.4 cells field^{-1}) and significant decrease in the leukocyte rolling velocity (68.8 ± 19.0 vs 157.1 ± 35.3 μm s^{-1}). Rolipram pretreatment provoked significant reductions in leukocyte responses elicited after 60 min LPS superfusion, inhibiting leukocyte rolling flux, adhesion and emigration by 91, 95 and 97% respectively. Again, decreases in leukocyte rolling velocities elicited by LPS were totally reversed by administration of rolipram (Figure 2).

Figure 3 shows the effect of rolipram on subacute LPS-induced leukocyte-endothelial cell interactions. After 4 h i.p. injection of 5 ml of 0.2 μg ml^{-1} LPS, significant increases in leukocyte rolling flux (171.3 ± 16.4 vs 29.8 ± 8.1 cells min^{-1}), adhesion (33.7 ± 1.6 vs 3.0 ± 0.9 cells 100 μm^{-1}) and emigration (22.0 ± 3.2 vs 1.3 ± 0.3 cells field^{-1}) and significant decreases in the leukocyte rolling velocity (21.3 ± 1.5 vs 81.6 ± 14.6 μm s^{-1}) were detected vs values obtained in the saline treated group. Rolipram pretreatment significantly reduced LPS-induced leukocyte rolling flux, adhesion and emigration by 51, 71 and 81% respectively after 4 h exposure to LPS (Figure 3) and significantly increased the reduction in the leukocyte rolling velocity elicited by LPS. None of these treatments had significant effects on circulating leukocyte counts. The values obtained were $13.2\pm2.9\times10^6$ cells ml^{-1} after 4 h saline i.p. injection, $13.1\pm2.7\times10^6$ cells ml^{-1} after 4 h LPS i.p. injection in animals pretreated with rolipram vehicle and $15.2\pm5.9\times10^6$ cells ml^{-1} after 4 h LPS i.p. injection in animals pretreated with rolipram ($n=6$ for each group).

Table 1 and 2 summarise the results obtained for MABP and shear rate prior to (0 min), 60 min following PAF or LPS superfusion and after 4 h exposure to LPS, in animals untreated and pretreated with rolipram. MABP and shear rate were unaffected by rolipram administration.

Immunohistochemical experiments revealed that when the mesenteric tissue was subjected to 60 min buffer superfusion or 4 h saline exposure, no increase in P-selectin, E-selectin or VCAM-1 expression was observed and ICAM-1 constitutive expression was the unique detected (Figures 4 and 5). Interestingly, whilst rolipram decreased P-selectin expression in animals suffused with PAF for 60 min, constitutive ICAM-

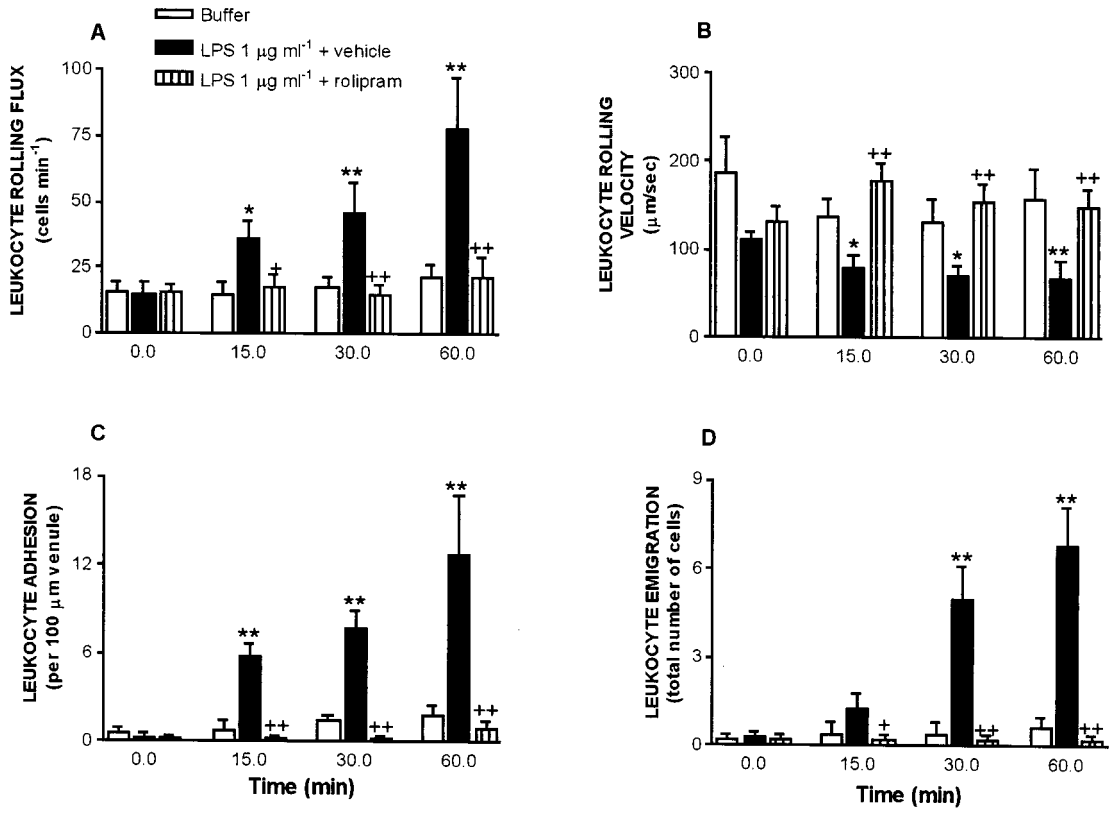

Figure 2 Effect of rolipram on acute LPS-induced leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C) and leukocyte emigration (D) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with buffer ($n=5$) or with LPS (1 μg ml^{-1}) in animals untreated (rolipram vehicle, $n=5$) or pretreated with rolipram (8 mg kg^{-1} i.p., $n=6$). Results are represented as mean$\pm$s.e.mean. $^{*}P<0.05$ or $^{**}P<0.01$ relative to values in the buffer group. $^{+}P<0.05$ or $^{++}P<0.01$ relative to the LPS untreated group.

1 expression was unaffected by this treatment (Figure 4). Furthermore, after 4 h LPS exposure significant increases in P-selectin, E-selectin, ICAM-1 and VCAM-1 expression were detected (Figure 5). However only P- and E-selectin expression were abrogated by rolipram preadministration, remaining ICAM-1 and VCAM-1 expression unaltered by this treatment (Figure 5).

Finally, to investigate whether rolipram-induced effects are mediated through increases in cyclic AMP levels, the plasma cyclic AMP levels were measured after 4 h of saline or LPS administration in rolipram- or vehicle-treated animals. Cyclic AMP levels in plasma samples of saline-treated animals were 54.0 ± 4.8 pmol ml^{-1}, similar to those found in LPS-injected pretreated with rolipram vehicle (58.9 ± 1.6 pmol ml^{-1}) whereas a significant increase in cyclic AMP levels was found in animals i.p. injected with LPS and pretreated with rolipram 8 mg kg^{-1} (79.1 ± 3.1 pmol ml^{-1}; $n=5$ for each group).

Discussion

Elevated cyclic AMP levels can be attained by either activating adenylate cyclase to increase synthesis or by inhibiting metabolism via PDE. The present study is the first to characterize systematically *in vivo* the effect of rolipram, a selective PDE-4 inhibitor, on CAM expression in leukocyte recruitment to the same tissue, under acute and subacute inflammatory conditions. In this regard, rolipram pretreatment was able to abolish totally the leukocyte-endothelial cell interactions elicited by PAF or LPS after 60 min superfusion in the rat mesenteric microvasculature. Interestingly, when the mesenteric tissue was exposed to LPS for 4 h, although leukocyte responses were significantly reduced by rolipram pretreatment, they were not completely abrogated. To characterize the endothelial CAMs downregulated by rolipram, immunohistochemical studies were carried out. Rolipram inhibited both P- and E-selectin expression in the stimulated venules under investigation; however, VCAM-1 and ICAM-1 endothelial expression were unaffected by this pretreatment. Furthermore, the effects exerted by rolipram seemed to be mediated through increases in cyclic AMP levels since enhanced amounts of this cyclic nucleotide were detected in plasma samples of animals pretreated with rolipram. Therefore, the present study demonstrates for the first time, to our knowledge, that rolipram selectively inhibits both P- and E-selectin expression *in vivo*, and consequently leukocyte recruitment. Conversely, this treatment did not affect the changes in different haemodynamic parameters caused by PAF or LPS superfusion for 60 min or by 4 h exposure to the latter.

Inhibition of leukocyte recruitment by PDE-4 inhibitors has been extensively demonstrated in several *in vivo* studies (Howell *et al*., 1995; Elwood *et al*., 1995; Ortiz *et al*., 1996;

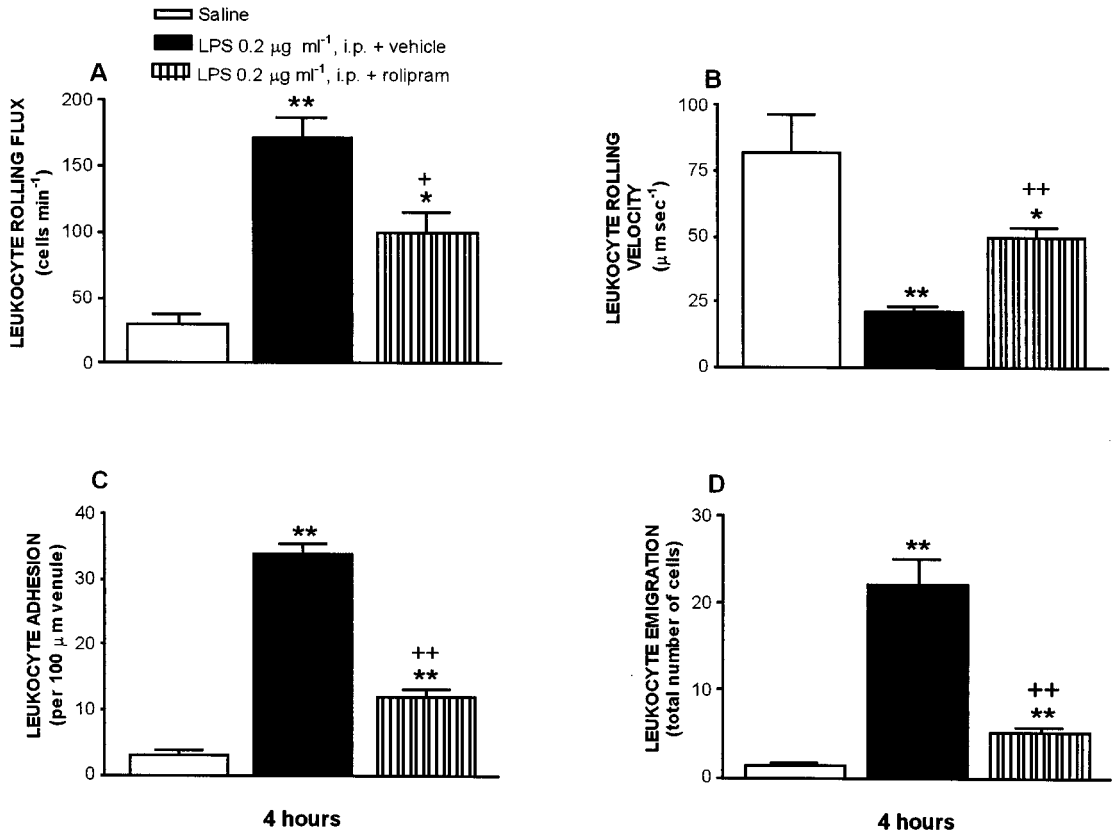

Figure 3 Effect of rolipram on subacute LPS-induced leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C) and leukocyte emigration (D) in rat mesenteric postcapillary venules. Parameters were measured 4 h after i.p. injection of saline ($n=5$) or 5 ml of LPS (0.2 μg ml^{-1}) in animals untreated (rolipram vehicle, $n=6$) or pretreated with rolipram (8 mg kg^{-1} i.p., $n=6$). Results are represented as mean$\pm$s.e.mean. *$P<0.05$ or **$P<0.01$ relative to saline group. +$P<0.05$ or ++$P<0.01$ relative to the LPS untreated group.

Table 1 Haemodynamic parameters in untreated and rolipram-treated animals before (0 min) and after (60 min) PAF (0.1 μM) or LPS (1 μg ml^{-1}) superfusion

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
Buffer	122.5$\pm$7.5	128.3$\pm$6.1	677.3$\pm$103.7	731.1$\pm$40.3
PAF (vehicle)	115.8$\pm$4.2	121.7$\pm$3.4	638.1$\pm$72.5	711.8$\pm$123.0
PAF (rolipram)	111.6$\pm$5.2	112.9$\pm$4.1	843.1$\pm$60.7	743.4$\pm$42.9
LPS (vehicle)	112.1$\pm$11.3	99.6$\pm$5.5	611.5$\pm$59.5	750.0$\pm$83.3
LPS (rolipram)	119.4$\pm$3.1	122.7$\pm$4.0	784.7$\pm$111.8	854.2$\pm$116.0

Values are mean$\pm$s.e.mean. No significant changes among the different groups were observed.

Table 2 Haemodynamic parameters in untreated and rolipram-treated animals after 4 h buffer and 5 ml of LPS (0.2 μg ml^{-1}) exposure

	MABP (mmHg)	*Shear rate* (s^{-1})
Treatment	*4 h*	*4 h*
Buffer	108.9$\pm$16.6	774.3$\pm$181.7
LPS (vehicle)	117.2$\pm$5.9	606.7$\pm$22.2
LPS (rolipram)	123.3$\pm$4.7	698.5$\pm$50.1

Values are mean$\pm$s.e.mean. No significant changes among the different groups were observed.

Miotla *et al*., 1998). Despite these findings, the adhesive mechanisms involved in these leukocyte responses are not precisely known. In this context, most of the studies carried out regarding this possibility have used *in vitro* models and contradictory findings have been encountered based on either the origin of the endothelial cells used or the stimulus employed to provoke leukocyte activation. For example, Pober *et al*. (1993) demonstrated that combination treatment with the adenylate cyclase stimulator forskolin and the non-specific PDE inhibitor isobutyl methylxanthine, suppressed the induction by cytokines of E-selectin and VCAM-1. In contrast, Morandini *et al*. (1996) showed that, while rolipram significantly suppressed the expression and release of E-selectin in TNF-α-stimulated human umbilical vein endothelial cells (HUVECs), when combined with forskolin, it had no effect on VCAM-1 expression. Similarly, Blease *et al*. (1998) using human lung microvascular endothelial cells (HLMVECs) found a significant reduction in TNF-α-induced

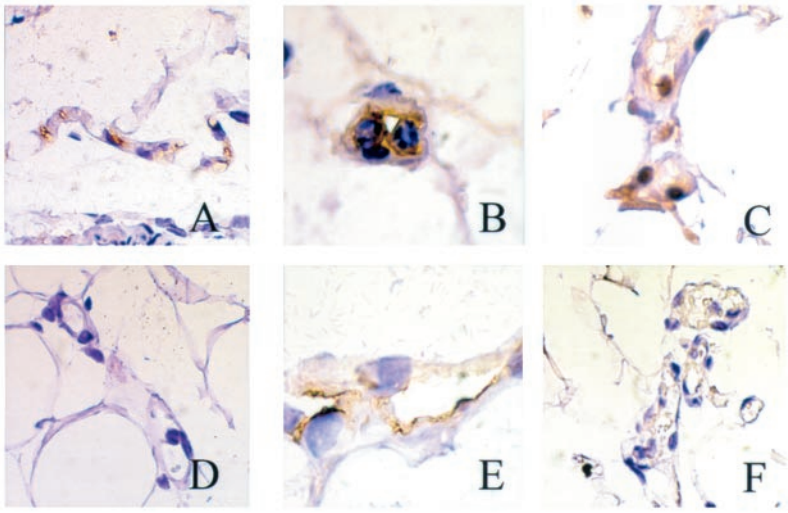

Figure 4 Representative photomicrographs of rat mesenteric venules showing immunolocalization of ICAM-1 and P-selectin expression in animals untreated and pretreated with rolipram after acute PAF superfusion. ICAM-1 expression after buffer (A) and PAF 60 min superfusion in the untreated group (B) and rolipram pretreated group (C). P-selectin expression after buffer (D) and PAF 60 min superfusion in the untreated group (E) and rolipram pretreated group (F). Brown reaction product indicates positive immunoperoxidase localization for both CAMs on the vascular endothelium. All four panels are lightly counterstained with haematoxylin and have the same magnification ($\times 400$). Results are representative of $n = 5$ experiments with each treatment.

E-selectin expression with a combination of rolipram and salbutamol, whereas no effect on ICAM-1 and VCAM-1 expression was detected. In fact, VCAM-1 expression was only affected when a combination of a PDE-4 and PDE-3 inhibitors were used.

The present study strongly suggests that the effects displayed *in vitro* do not necessarily correlate with those detected in *in vivo* studies. In fact, we have shown that acute PAF and LPS-induced leukocyte-endothelial cell interactions were completely blocked by rolipram. Therefore, it seems likely that, *in vivo*, downregulation of adhesion molecules other than E-selectin or VCAM-1 may be involved in the inhibitory responses observed in the present study. In this context, one possible candidate is P-selectin. Indeed, rolipram inhibition of acute leukocyte responses appears to be primarily mediated through a decreased expression of P-selectin on the endothelial cell surface, since up-regulation of this CAM was not detected in animals pretreated with this PDE-4 inhibitor. Supporting this possibility, other studies have also shown that cyclic AMP elevating agents can reduce P-selectin expression and release in stimulated platelets (Konstantopoulos *et al*., 1998). In addition, we have recently found that cyclic AMP elevating agents which activate adenylate cyclase such as forskolin, salbutamol and iloprost as well as the nonspecific PDE inhibitor, theophylline, or the selective PDE-4 inhibitor, rolipram, are potent inhibitors of angiotensin II-induced leukocyte endothelial cell interactions *in vivo* regardless of the mechanism employed to elevate cyclic AMP levels (Alvarez *et al*., 2001b). The effects observed seemed to be mediated through endothelial P-selectin down-regulation, since angiotensin-II-induced leukocyte recruitment is primarily dependent on increased endothelial P-selectin expression (Piqueras *et al*., 2000). Nevertheless, although the inhibition of P-selectin expression may delay the onset of leukocyte adhesion and emigration, a direct effect of rolipram on leukocyte surface adhesion molecule expression cannot be excluded, since downregulation of β_2-integrins by this PDE-4 inhibitor has been extensively demonstrated in several *in vitro* studies (Derian *et al*., 1995; Teixeira *et al*., 1996; Berends *et al*., 1997; Santamaria *et al*., 1997).

Also surprising was the increase encountered in leukocyte rolling flux upon PAF superfusion. In this regard, although some authors could only find a role for PAF in leukocyte adhesion (Bienvenu *et al*., 1993; Gaboury *et al*., 1995), others have found that PAF superfusion causes significant increases in leukocyte rolling flux within 30 min of superfusion or that increases in the flux of rolling leukocytes induced by different mediators can be significantly diminished by PAF receptor antagonist pretreatment (DeLano *et al*., 1997; Panes *et al*., 1995; Silvestro *et al*., 1994; Vergnolle, 1999). Therefore, we suggest that PAF can not only increase CD11/CD18 integrin expression on the leukocyte cell surface, but also act on the endothelial cell inducing rapid P-selectin expression as demonstrated in this and in previous studies (DeLano *et al*., 1997; Silvestro *et al*., 1994). Whether this effect is direct or indirect through the release of preformed inflammatory mediators from platelets or mast cells remains to be determined.

In our model of LPS-induced inflammation, while the early (acute) increase in the leukocyte recruitment is P-selectin-dependent, under subacute (4 h) inflammatory conditions P-selectin-independent mechanisms were observed. This latter phase of leukocyte recruitment was only partly reduced by rolipram. Immunohistochemical analysis revealed that only P- and E-selectin expression were completely inhibited, while up-regulation of ICAM-1 and VCAM-1 were not affected by this treatment. Although it was reported that the contribution of E-selectin to leukocyte recruitment in this vascular bed is not important (Johnston *et al*., 1997), we have found an increased expression of this selectin. Consistent with our finding, E-selectin was found relevant in leukocyte recruitment to dermal tissues (Walter & Issekutz, 1997) and in LPS-

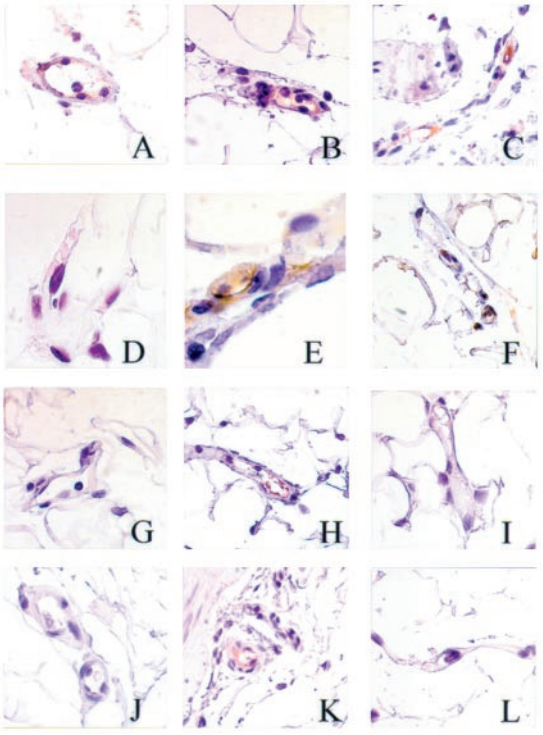

Figure 5 Representative photomicrographs of rat mesenteric venules showing immunolocalization of ICAM-1, VCAM-1, P-selectin and E-selectin, expression in animals untreated and pretreated with rolipram after subacute LPS exposure. ICAM-1 expression after 4 h saline (A) or LPS exposure in the untreated group (B) and rolipram pretreated group (C). VCAM-1 expression after 4 h saline (D) or LPS exposure in the untreated group (E) and rolipram pretreated group (F). P-selectin expression after 4 h saline (G) or LPS exposure in the untreated group (H) and rolipram pretreated group (I). E-selectin expression after 4 h saline (J) or LPS exposure in the untreated group (K) and rolipram pretreated group (L). Brown reaction product indicates positive immunoperoxidase localization for all CAMs on the vascular endothelium. All panels are highly counterstained with haematoxylin and have the same magnification ($\times 400$). Results are representative of $n=6$ experiments with each treatment.

induced leukocyte recruitment to the murine lung (Henriques *et al*., 1996). Therefore, it seems likely that the remaining leukocyte rolling detected after 4 h LPS-exposure, may be both L-selectin and α_4-integrin-dependent. Indeed, Johnston *et al*. (1997) demonstrated that the increase in leukocyte rolling flux elicited by LPS was entirely dependent on P-selectin expression during the first 90 min of superfusion and L-selectin or the α_4-integrin played a prominent role in the subsequent phase (90–180 min) since the use of antibodies against either of these CAMs was necessary to abrogate this leukocyte-endothelial cell interaction. In that study, a sequential pattern was suggested, in which leukocytes may use L-selectin to tether to the endothelium and then roll *via* the α_4-integrin. In fact, L-selectin has been demonstrated to mediate the initial attachment of leukocyte to endothelium *in vitro* and *in vivo* (Johnston *et al*., 1996; Lawrence *et al*., 1994), and the absence of α_4-integrin-dependent leukocyte rolling in the presence of L-selectin antibody supported this sequential pattern. The overlapping requirements for L-selectin and the α_4-integrin were consistent with the adhesion cascade reported under shear conditions for eosinophils and monocytes (Sriramarao *et al*., 1994; Luscinskas *et al*., 1994). L-selectin appears to mediate the initial tethering of these cells to the endothelium while the α_4-integrin mediates the transition from rolling to firm adhesion. Hence, since rolipram has no effect on VCAM-1 expression and the main leukocyte ligands for VCAM-1 are the α_4-integrins, $\alpha_4\beta_1$ and $\alpha_4\beta_7$, and since rolipram in fact sustains L-selectin expression on leukocyte surface (i.e. it inhibits activation-induced L-selectin shedding), the remaining leukocyte rolling flux detected after 4 h LPS exposure in animals treated with this PDE-4 inhibitor may be explained by the contribution of these two adhesion pathways.

Of interest is the fact that even though rolipram caused a significant decrease in the leukocyte rolling flux elicited after 4 h LPS exposure, dramatic effects on leukocyte adhesion were detected at this time point. Although rolipram administration had no effect on ICAM-1 expression, its ability to decrease β_2-integrin expression on leukocyte cell surface (Derian *et al*., 1995; Teixeira *et al*., 1996; Berends *et al*., 1997; Santamaria *et al*., 1997), may account for the further reduction observed in leukocyte adhesion. Indeed, β_2-integrins can interact with endothelial cell ligand(s) other than ICAM-1 (Staunton *et al*., 1989; Lo *et al*., 1989; Stacker & Springer, 1991). Therefore, the findings in the present study suggest that the decrease in leukocyte adhesion and emigration induced by rolipram under subacute inflammatory conditions can be partly explained by its ability to down-regulate both P- and E-selectin expression on the endothelial cell surface together with decreased β_2-integrin expression on leukocytes as found in other studies (Derian *et al*., 1995; Teixeira *et al*., 1996; Berends *et al*., 1997; Santamaria *et al*., 1997).

In conclusion, in the present study we have provided evidence that rolipram is a potent *in vivo* inhibitor of leukocyte-endothelial cell interactions in both acute and subacute inflammatory conditions. The effects observed are likely mediated through P- and E-selectin downregulation on the endothelial cell surface, and through decrease in β_2-integrin expression on the leukocyte cell surface. Thus, PDE-4 inhibitors can impair leukocyte infiltration associated with different inflammatory disorders acting on different adhesive pathways.

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