

Inhibition of PAF-induced expression of CD11b and shedding of L-selectin on human neutrophils and eosinophils by the type IV selective PDE inhibitor, rolipram

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ABSTRACT: We quantitatively determined whether the selective phosphodiesterase (PDE) inhibitor, rolipram, inhibits changes in the adhesion molecules CD11b and L-selectin on platelet-activating factor (PAF)-stimulated human neutrophils and eosinophils *in vitro*.

Incubations were performed in human whole blood obtained from healthy volunteers, to restrict activation by purification procedures and to simulate *in vivo* conditions, in which different cell types may interact, more closely. Receptor expression was measured after fixation of cells, using monoclonal antibodies and flow cytometry.

Concentration-dependent inhibition of the PAF-induced CD11b expression and L-selectin shedding for neutrophils and eosinophils was observed with rolipram, dibutyryl cyclic adenosine monophosphate (cAMP), prostaglandin E₂ (PGE₂), and isoproterenol. However, these inhibitions did not exceed 50%. Preincubation with rolipram (10⁻⁸ M) and subsequent incubation with isoproterenol (0.5×10⁻⁸ M) or PGE₂ (10⁻⁸ M) induced a cumulative, but not synergistic, effect. Using the combination of rolipram with isoproterenol or PGE₂, inhibition of PAF-induced L-selectin shedding from eosinophils was as high as 71±28 and 67±21%, respectively. Other inhibitions were below 50%.

In conclusion, rolipram inhibits CD11b expression and L-selectin shedding of platelet-activating factor-stimulated neutrophils and eosinophils in whole blood in a concentration-dependent fashion. Inhibitions did not exceed 50%, even at high concentrations. The inhibition of platelet-activating factor induced shedding of L-selectin from eosinophils with a combination of rolipram and prostaglandin E₂ or isoproterenol, however, was found to be approximately 70%. Inhibition of rolling adhesion of eosinophils may, therefore, be a mode of action of type IV phosphodiesterase inhibitors.

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Extravasation and infiltration of neutrophils and eosinophils into tissues occurs in various inflammatory lung diseases [1–3]. Local activation of these cells and release of reactive oxygen species, lipid mediators, and toxic granule constituents subsequently contributes to the pathological features of these disorders [4, 5]. Therapeutic intervention aimed at inhibiting granulocyte infiltration may reduce the inflammatory response and limit tissue damage.

Infiltration of granulocytes is orchestrated by a number of receptors belonging to different distinct families of adhesion molecules [6, 7]. During the initial step of adhesion and transendothelial migration, granulocytes roll along the vascular wall [8]. This step is mediated by reversible binding of L-selectin (lectin adhesion molecule-1 (LECAM-1), Leu8) on the granulocyte to counter structures on endothelial cells. This initial step is fol-

lowed by a firmer adhesion. Mac-1 (CD11b/CD18, CR3) is one of the molecules involved in this process. This molecule belongs to the β₂-family of integrins and may bind to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells. Upon activation of the granulocyte by mediators bound to the endothelial cell surface, L-selectin is rapidly shed from the cell surface, and Mac-1 is concomitantly increased by release from intracellular stores [9, 10]. One of the activating agents produced by endothelial cells is platelet-activating factor (PAF) [11]. The increase of CD11b expression and concomitant shedding of L-selectin is not only observed *in vitro* [12, 13]; a number of studies have shown increased Mac-1 expression and decreased L-selectin expression on extravasated neutrophils and eosinophils *in vivo* [2, 14, 15].

Animal studies have shown that infiltration of neutrophils and eosinophils can be inhibited by rolipram

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[16–18]. Rolipram inhibits phosphodiesterase (PDE) type IV, which hydrolyses cyclic adenosine monophosphate (cAMP) into the biologically inactive form 5'-adenosine monophosphate (5'-AMP) [19]. Therefore, inhibition of PDE increases intracellular cAMP [20], which may exert various inhibitory effects, such as relaxation of airway smooth muscle and inhibition of mediator release by inflammatory cells [20–23]. Other PDE isoenzymes have been described, but type IV is the predominant type present in neutrophils and eosinophils [20, 23]. PDE inhibitors may act synergistically with receptor-dependent activators of adenylyl cyclase to inhibit polymorphonuclear leucocyte activation *in vitro* [23–25]. Currently, PDE IV inhibitors are considered one of the most promising antiasthma drugs for the future [26]. In this context, the effects of these compounds on the eosinophil is of interest.

Rolipram may inhibit rolling, adhesion and transmigration of granulocytes by direct effects on receptor numbers. In a recent study from DERIAN *et al.* [27], it was shown that rolipram attenuated neutrophil-endothelial adhesion by 80%. Inhibition of neutrophil CD11b surface expression was observed in that study but not quantified.

In the present study, we quantitatively determined whether rolipram can inhibit the shedding of L-selectin, involved in rolling, as well as the upregulation of CD11b, involved in firm adhesion. Since purification of neutrophils and eosinophils has been shown to modulate surface expression of CD11b [28–30], a whole blood system was used. This also allowed for interactions between different cell types in blood, thus more closely approaching the situation *in vivo*. Hence, the results may be more indicative for the effects of rolipram *in vivo* than results of experiments with isolated granulocytes. In addition, we studied whether isoproterenol and prostaglandin E₂ (PGE₂) act synergistically on adhesion receptor inhibition by rolipram.

Materials and methods

Subjects and reagents

Blood was drawn from healthy volunteers, who all gave their informed consent. Eosinophil counts were normal, between 40 and 437 cells· μL^{-1} of blood.

PAF (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.9% NaCl, containing 2.5% human serum albumin (HSA) at a concentration of 200 μM , before final dilution in Hank's balanced salt solution (HBSS, Gibco BRL Paisley, Scotland). Isoproterenol and dibutyryl cAMP (Sigma) were directly dissolved in HBSS. PGE₂ (Sigma) was dissolved in absolute ethanol to obtain a stock solution of 2×10^{-2} M, and then diluted in HBSS to the final concentrations indicated. Rolipram, a gift from Schering (Berlin, Germany), was first dissolved in dimethyl sulphoxide (DMSO), which resulted in a stock of 10^{-1} M. Phosphate-buffered saline (PBS) containing 2.5% (v/v) of a heat-inactivated bovine albumin solution (200 g·L⁻¹ with 1 g·L⁻¹ sodium azide; Organon Teknika, Boxtel, The Netherlands) was used as a washing solution. Human AB-serum was obtained from the Laboratory of The Netherlands Red Cross Blood

Bank (Groningen, The Netherlands). Formaldehyde fixative (37%; Merck, Darmstadt, Germany) was diluted in PBS to acquire a final concentration of 0.1%.

Monoclonal antibodies

Antibodies against CD11b, L-selectin (Leu8) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (GAM-FITC) were purchased from Becton Dickinson (Mountain View, CA, USA). Mouse isotype control antibodies (immunoglobulin 2a (IgG2a)) were supplied by the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Cell stimulation

In order to investigate whether the effects of rolipram on receptor expression could be related to an increase in intracellular cAMP, inhibition by other cAMP elevating agents on PAF-induced adhesion receptor expression was also tested. This was done in a number of experiments by incubating cells in the presence of buffer or 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M of PGE₂ or isoproterenol. In addition, the effect of preincubation with 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} M of dibutyryl cAMP was studied. Concentration curves were drawn for each separate experiment. For rolipram, the concentration that induces 20% inhibition (IC₂₀) was determined from these curves.

Cells were stimulated and labelled in whole blood, according to a slightly modified procedure described previously [31]. Briefly, ethylenediamine tetra-acetic acid (EDTA)-anticoagulated blood was washed twice with excess PBS at room temperature. In order to determine *in vivo* CD11b expression as a control, some blood samples were fixed with 0.1% formaldehyde directly after collection, before washing. After washing, the cells were resuspended in PBS to reconstitute the original volume. From this suspension, 100 μL samples were stimulated with buffer or PAF for 15 min at 37°C, in a total volume of 500 μL . PAF was used, since it is a stimulus for neutrophils as well as eosinophils, and may play a role in asthmatic inflammation [32].

The effects of the receptor-dependent inhibitors, isoproterenol and PGE₂, were tested by simultaneous incubation with PAF. To allow intracellular diffusion, the inhibitory effect of dibutyryl cAMP was studied by preincubation for 15 min at 37°C before incubation with PAF. The effect of rolipram was also tested by preincubation for 15 min, and subsequent incubation with PAF with or without PGE₂ or isoproterenol. The incubations were stopped by adding 2 mL of ice-cold washing buffer. After centrifugation at 1,000×g for 2 min, the cells were fixed with 0.1% formaldehyde for 10 min and stained for flow cytometry.

Effects of rolipram in combination with PGE₂ or isoproterenol

PDE inhibitors, such as rolipram, may act synergistically with receptor-dependent activators of the adenylyl cyclase system *in vitro* [23–25]. Therefore, the effect

of rolipram was studied in the presence of a receptor-coupled stimulus of the adenylyl cyclase system. For this purpose, 10^{-8} M of rolipram was used, because this concentration induced little direct inhibition of PAF-induced receptor expression. Whole blood was preincubated with rolipram, and subsequent PAF-stimulation was performed in the presence or absence of 0.5×10^{-8} M isoproterenol or 10^{-8} M PGE₂. These concentrations of isoproterenol and PGE₂ result in approximately half of the maximum inhibition of the PAF response, as estimated from the concentration curves in figure 1a and b. Since the eosinophil was the main cell type of interest, the curves for eosinophils were used for this purpose.

Immunofluorescence staining

After fixation, the cells were labelled for 15 min at room temperature with 10 μ L of anti-CD11b, anti-L-selectin or control antibodies of the same mouse isotype

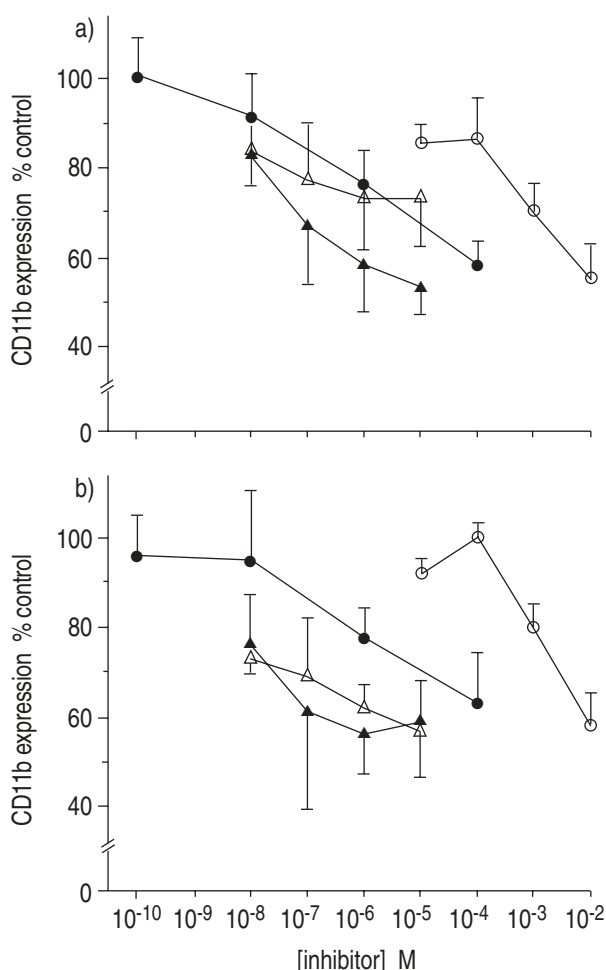


Fig. 1. — Effect of different concentrations of prostaglandin E₂ (PGE₂) (n=3), isoproterenol (n=4), dibutyryl cyclic adenosine monophosphate (cAMP) (n=3) or rolipram (n=5) on the PAF-induced (10^{-7} M) CD11b expression of: a) neutrophils; and b) eosinophils, in whole blood. Results are presented as a percentage of the response with PAF in the absence of cAMP modulating agents (% of control). Data are presented as mean \pm SEM. No effects of cAMP modulators were seen in the absence of PAF (not shown). \blacktriangle : PGE₂; \triangle : isoproterenol; \bullet : rolipram; \circ : dibutyryl cAMP. PAF: platelet-activating factor.

(IgG2a). This was done in the presence of 10 μ L of diluted human AB-serum (20%), in order to prevent non-specific binding of antibody. After lysis of red blood cells with lysis buffer (155 mmol·L⁻¹ NH₄Cl, 10 mmol·L⁻¹ KHCO₃, 0.1 mmol·L⁻¹ EDTA), each sample was incubated with 50 μ L of GAM-FITC (20% v/v in washing buffer), in the presence of 5% AB-serum, for 15 min at room temperature in the dark. After washing, the cells were resuspended in washing buffer and kept at 4°C in the dark until analysed. Flow cytometric analysis was performed within 4 h after staining.

Flow cytometry

Flow cytometric measurements were performed with a fluorescence-activated cell sorter (Becton-Dickinson FACStar). Fluorescence gain was standardized with Quick Cell 3 beads (Flow Cytometry Standards Co., San Juan, Puerto Rico). Neutrophils and eosinophils were discriminated by means of depolarized orthogonal light scattering, as described previously [28, 31, 33]. In samples with low eosinophil numbers, the threshold for depolarized light scatter was increased in order to exclude neutrophils from the 10,000 events that were collected. In this way, higher numbers of eosinophils could be obtained during data collection. Data on neutrophils were collected by a second measurement of the same sample using a normal threshold. Evaluation of mean specific fluorescence on neutrophils and eosinophils was performed using a personal computer (Hewlett Packard) and the Lysis program (Becton Dickinson). During evaluation, neutrophils and eosinophils were gated based on forward scatter/sideward scatter (FSC/SSC), and further discriminated by SSC and depolarized light scattering.

Expression of specific receptors is presented as mean fluorescence intensity (MFI) (\pm SEM), after subtraction of the fluorescence with control antibody. The percentage control in the presence of a cAMP-elevating agent was calculated as percentage of the response by PAF alone.

Statistical analysis

Differences between various incubation conditions were evaluated using Student's t-test for paired data. A p-value less than 0.05 was considered significant.

Results

Effect of PAF on receptor expression

A concentration of 10^{-7} M PAF did not cause maximal CD11b expression on neutrophils and eosinophils, since 10^{-6} M was able to induce an additional increase (fig. 2a). In order to ensure sensitivity to the effects of the inhibitory compounds, a submaximal concentration of PAF was selected. Since 10^{-6} M induced more CD11b expression than 10^{-7} M (fig. 2a), the latter concentration

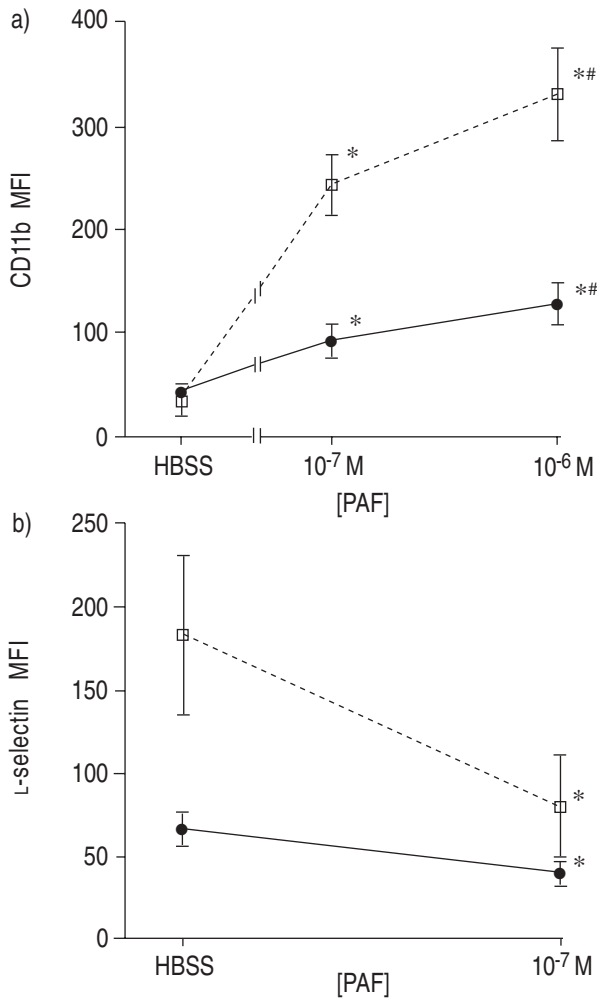


Fig. 2. — a) Effect of PAF (—□—) versus buffer (HBSS) (—●—) on CD11b expression of neutrophils and eosinophils. Whole blood was incubated with 10^{-7} or 10^{-6} M of PAF for 15 min at 37°C . Subsequently, CD11b expression was determined by flow cytometry after labelling with anti-CD11b and GAM-FITC, as described in Materials and methods. Results are presented as mean \pm SEM ($n=7$). b) Similarly, the effect of 10^{-7} M of PAF on L-selectin expression ($n=8$) was determined. PAF: platelet-activating factor; HBSS: Hank's balanced salt solution; GAM-FITC: fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin; MFI: mean fluorescence intensity. *: $p<0.05$ vs HBSS, Student's paired t-test; #: $p<0.05$ vs 10^{-7} M PAF, Student's paired t-test.

was chosen. The relative increases of CD11b on neutrophils and eosinophils induced by this concentration of PAF were 615 and 110%, respectively. In addition, a significant decrease ($p<0.05$) of L-selectin on neutrophils (-57%) and eosinophils (-41%) could be observed after incubation with this concentration of PAF (fig. 2b). For both receptors, the effects of PAF were more marked on neutrophils than on eosinophils. CD11b expressions in samples incubated with HBSS were not significantly different from directly fixed blood samples. CD11b expression on neutrophils and eosinophils in directly fixed blood samples was 37 ± 9 and 61 ± 8 ($n=7$), respectively. After incubation with HBSS for 15 min at 37°C , these values were 36 ± 9 and 59 ± 6 , respectively. This indicates that no activation was induced by handling of the cells.

Effect of single cAMP modulators on CD11b expression

From separate experiments with PGE_2 , isoproterenol, dibutyryl cAMP and rolipram concentration curves were drawn. In most experiments, the maximum inhibition observed was less than 50%.

As shown in figure 1a and b, PGE_2 was the most potent inhibitor of PAF-induced CD11b expression on neutrophils and eosinophils, reducing the PAF responses by 47 ± 6 and $41\pm 9\%$, respectively ($n=3$). Significant ($p<0.05$) inhibition was observed at concentrations of PGE_2 higher than 10^{-6} M.

Isoproterenol also significantly reduced PAF-induced CD11b expression ($p<0.05$). However, in neutrophils, it was less potent than PGE_2 , and 10^{-5} M isoproterenol resulted in a maximum inhibition of only $27\pm 11\%$ (fig. 2a). The curve for neutrophils was flatter than the curve for eosinophils. In eosinophils, the maximum inhibition of the response by isoproterenol was comparable to the inhibition by PGE_2 ($43\pm 11\%$). The difference between neutrophils and eosinophils for the maximum effect of isoproterenol in this limited number of experiments was, however, not significant. Dibutyryl cAMP was a much less potent inhibitor of the PAF-induced CD11b expression than PGE_2 , as the concentration curve was shifted to the right (fig. 1a and b). The maximal inhibition of neutrophils and eosinophils was comparable.

The PAF-induced CD11b expression on neutrophils and eosinophils was significantly inhibited by rolipram (fig. 1a and b; $n=5$). Although there was a trend for inhibition at 10^{-8} M rolipram, statistical significance was found at 10^{-6} M for both cell types. With the highest concentration of rolipram, 10^{-4} M, the inhibition of CD11b expression on neutrophils and eosinophils was 41 ± 5 and $37\pm 11\%$, respectively. Since 50% inhibition was not achieved, the concentration inducing 50% inhibition (IC_{50}) could not be determined, and the IC_{20} was determined instead. The IC_{20} s of rolipram for inhibition of CD11b expression were 10 ± 14 μM for neutrophils and 0.2 ± 0.4 μM for eosinophils.

Effect of single cAMP modulators on L-selectin expression

The PAF-induced L-selectin shedding on neutrophils and eosinophils was also inhibited by PGE_2 , isoproterenol, dibutyryl cAMP and rolipram.

PGE_2 was the most potent inhibitor of L-selectin shedding from neutrophils (fig. 3a). The maximum inhibition observed at 10^{-5} M was $52\pm 3\%$ for neutrophils and $37\pm 11\%$ for eosinophils (fig. 3a and b).

The maximum inhibition of PAF-induced L-selectin shedding from neutrophils by 10^{-5} M of isoproterenol was only $22\pm 7\%$ (fig. 3a). For eosinophils, however, it was comparable to the mean maximum inhibition by PGE_2 , $34\pm 24\%$ (fig. 3b). The difference between neutrophils and eosinophils was not statistically significant in these experiments.

Again, dibutyryl cAMP was the least potent inhibitor of PAF-induced L-selectin shedding (fig. 3a and b). Maximum inhibition of L-selectin shedding from neutrophils and eosinophils was 37 ± 10 and $29\pm 6\%$. Remarkably, the curve for neutrophils was much steeper than the curve for eosinophils.

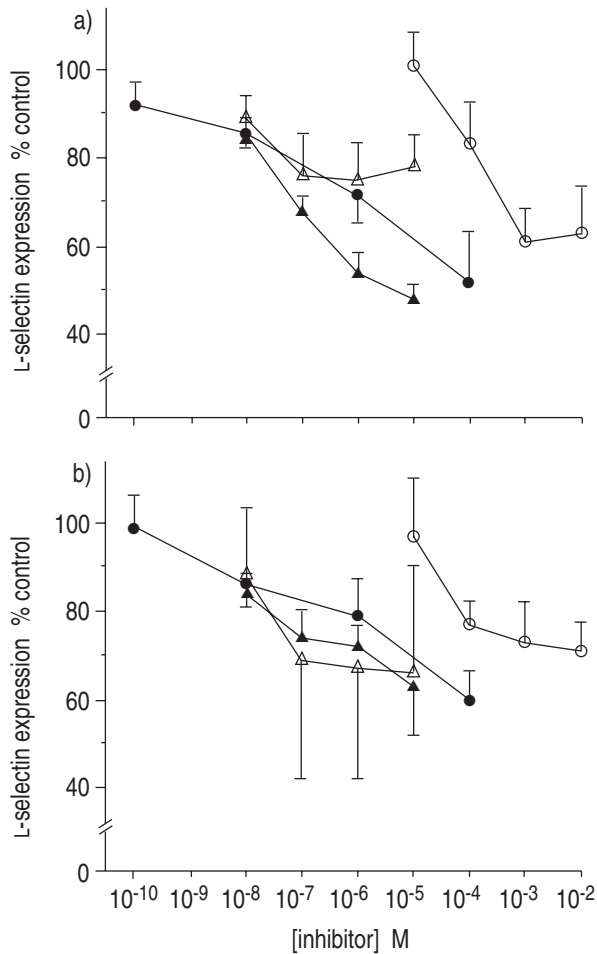


Fig. 3. – Effect of different concentrations of prostaglandin E₂ (PGE₂) (n=3), isoproterenol (n=4), dibutyryl cyclic adenosine monophosphate (cAMP) (n=3) or rolipram (n=5) on the PAF-induced (10⁻⁷ M) L-selectin expression of: a) neutrophils; and b) eosinophils, in whole blood. Results are presented as a percentage of the response with PAF in the absence of cAMP modulating agents (% of control). Data are presented as mean±SEM. No effects of cAMP modulators were seen in the absence of PAF (not shown). —▲— : PGE₂; —△— : isoproterenol; —●— : rolipram; —○— : dibutyryl cAMP. PAF: platelet-activating factor.

Small but statistically significant inhibition of PAF-induced L-selectin shedding from neutrophils and eosinophils was found with 10⁻¹⁰ and 10⁻⁸ M of rolipram, respectively. Maximum inhibition of 48±11 and 41±6%, respectively, was observed with 10⁻⁴ M rolipram. This was comparable to the maximum inhibitions found with PGE₂. The IC₂₀s for rolipram on PAF-induced L-selectin shedding from neutrophils and eosinophils were 15±21 and 0.2±0.4 μM, respectively.

As shown in figure 4a, the combination of rolipram and isoproterenol or PGE₂ induced a statistically significantly (p<0.05) greater inhibition of PAF-induced CD11b expression than each inhibitor alone (n=7). This was observed for neutrophils as well as eosinophils. When the inhibition, calculated as 100 - % of control, with the combination of rolipram and isoproterenol or PGE₂ was compared with the sum of the inhibition by each agent separately, no differences were found. For instance, the sum of the inhibition of neutrophil CD11b expression by rolipram and isoproterenol was 40±11%. When rolipram and isoproterenol were combined in the

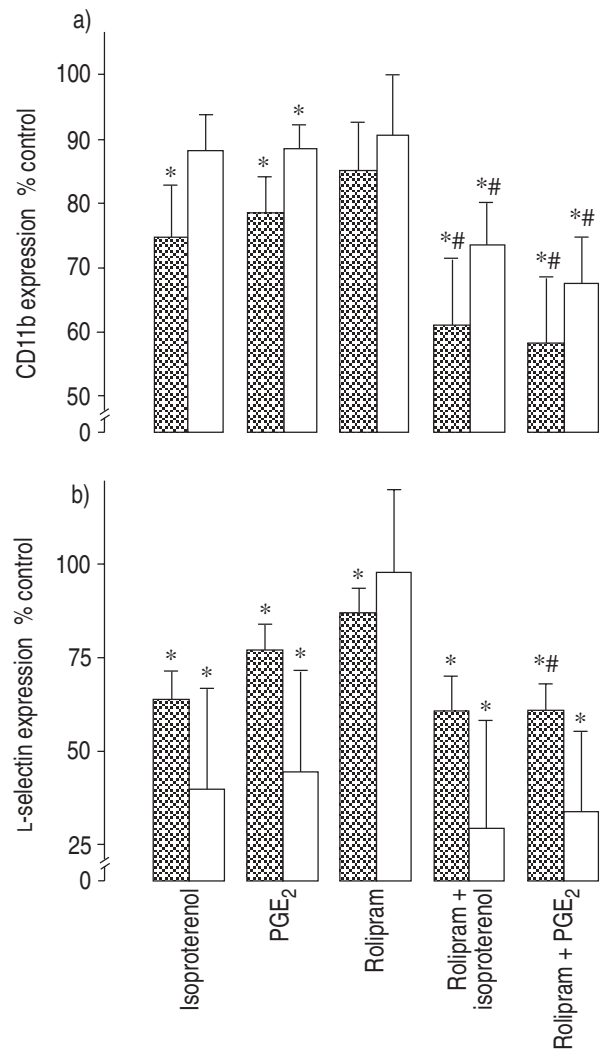


Fig. 4. – Effect of isoproterenol (0.5×10⁻⁸ M), prostaglandin E₂ (PGE₂) (10⁻⁸ M), rolipram (10⁻⁸ M) or combinations of these agents on: a) CD11b expression; and b) L-selectin expression of PAF-stimulated neutrophils or eosinophils (n=7). Results are expressed as a percentage of the response induced by PAF in the absence of inhibitors; a) PAF increased CD11b expression from 32±9 to 312±46 for neutrophils and from 47±6 to 148±20 for eosinophils (MFI mean±SEM). b) PAF decreased L-selectin expression from 177±18 to 72±10 for neutrophils and from 66±9 to 39±6 for eosinophils (MFI mean±SEM). No effects of inhibitors were seen in the absence of PAF (not shown). [hatched box] : neutrophils; [white box] : eosinophils. For definitions see legend to figure 1. *: p<0.05 vs PAF without inhibitor, Student's paired t-test; #: p<0.05 vs corresponding sample without rolipram, Student's paired t-test.

same experiment, 39±10% inhibition of the relative increase in CD11b was found. This indicates an additive response of rolipram and isoproterenol. Similar results were observed for PGE₂.

In the same experiments, the effect of rolipram and isoproterenol or PGE₂ was investigated on the PAF-induced decrease of L-selectin (fig. 4b). However, a significant effect of combining inhibitors was only found for rolipram and PGE₂ on neutrophils, and not on eosinophils. As had been found for CD11b, the sum of the single inhibitions by rolipram and PGE₂ was not significantly different from the inhibition observed when the agents were combined in the same sample. Again, this indicates an additive, but not synergistic, inhibition.

Remarkably, the combination of rolipram and isoproterenol or PGE₂ inhibited the L-selectin shedding from eosinophils by 71±28 and 67±21%, respectively. Although a trend could be observed, the additive effect of rolipram and isoproterenol on L-selectin was not significant.

Discussion

The present study demonstrates that rolipram inhibits PAF-induced CD11b increase and a concomitant L-selectin decrease on human neutrophils and eosinophils. The experiments have been performed in unseparated whole blood samples, so that interactions between cell types were similar to the *in vivo* situation, and less *in vitro* activation of granulocytes was induced by isolation procedures [29, 30]. An additional, but not synergistic, inhibitory effect on PAF-induced receptor expression was observed when rolipram was followed by an activator of the adenylyl cyclase.

NEELEY *et al.* [12] reported that 10⁻⁷ M PAF caused mean increases of CD11b expression of 35 and 43% in neutrophils and eosinophils, respectively. In the present study, these increases were as high as 615% in neutrophils and 110% in eosinophils. Similarly, the concomitant decrease of L-selectin expression on neutrophils was higher in the present study. Moreover, NEELEY *et al.* [12] observed that 10⁻⁸ M PAF caused changes in eosinophil CD11b expression similar to those caused by 10⁻⁷ M of PAF, suggesting a maximal effect at 10⁻⁸ M. In the present study, the CD11b expression continued to increase with 10⁻⁶ M of PAF. The differences in the magnitude of the response to PAF may be due to the fact that NEELEY *et al.* [12] purified cells using several washing steps, thereby inducing a higher basal CD11b expression [28–30]. Basal CD11b expression of neutrophils and eosinophils in the present study was not changed by incubation, as shown by the fact that no significant differences were found between buffer-incubated cells and directly fixed cells.

Adhesion and transmigration of granulocytes is not only regulated by receptor number but also by receptor avidity [34, 35]. DERIAN *et al.* [27] reported approximately 80% inhibition of N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced neutrophil adherence to endothelial cells by 1–10 µM rolipram. CD11b expression was also shown to be inhibited, but this was not analysed quantitatively. In the present study, however, it was shown that this inhibition does not exceed 50%, even with high concentrations of rolipram. When 1 µM rolipram was used, the inhibition of CD11b expression was only 20–25% for neutrophils and eosinophils. Therefore, it is very likely that rolipram and other cAMP modulators also inhibit receptor avidity as well as CD11b receptor number. However, stimulus specificity or additional actions on endothelial cells may also be involved in the disparity between the effects of rolipram on the expression of adhesion molecules and adhesion. Moreover, enlarging the magnitude of the response by using whole blood cells, as discussed above, may decrease the potency of inhibitors.

Although there was a trend for inhibition of CD11b expression at low concentrations of rolipram, significant inhibition was found at 10⁻⁶ and 10⁻⁴ M rolipram.

This is in accordance with the study by NIELSON *et al.* [23], in which significant inhibition of the respiratory burst of polymorphonuclear leucocytes was found at concentrations of rolipram greater than 10⁻⁶ M. The degree of inhibition observed at the highest concentrations is also similar to that observed for inhibition of respiratory burst in human neutrophils [23] and eosinophils [36]. DENT and co-workers [36] have reported an IC₅₀ of approximately 40 µM for inhibition of opsonized zymosan-induced superoxide production in eosinophils. From the curves shown in their paper, we estimated an IC₂₀ of 0.1 µM. The IC₂₀ for inhibition of PAF-induced adhesion receptor expression in the present study was 0.2 µM. Thus, inhibition of respiratory burst and adhesion receptor expression may occur at similar concentrations of rolipram.

L-selectin decrease was also observed to be inhibited by rolipram. Interestingly, significant inhibition of L-selectin shedding was found with concentrations of rolipram as low as 10⁻⁸ M. Furthermore, a combination of 10⁻⁸ M rolipram and 0.5×10⁻⁸ M isoproterenol or 10⁻⁸ M PGE₂ inhibited L-selectin shedding on eosinophils by 70%. Therefore, the initial rolling step in eosinophil infiltration may be equally or more sensitive to inhibition by cAMP modulators than CD11b-mediated adhesion. PDE inhibitors may, therefore, be more successful when applied systemically, thereby attenuating rolling of eosinophils in peripheral blood. This may explain why orally administered theophylline attenuates eosinophil infiltration in asthmatic patients, as has been described recently [37].

Rolipram has been shown to increase intracellular cAMP in neutrophils and eosinophils *via* inhibition of a cAMP-specific, cyclic guanosine monophosphate (cGMP)-insensitive PDE [20, 23]. Therefore, the inhibition of PAF-induced CD11b and L-selectin expression, described in the present study, is likely to be due to a rise in intracellular cAMP. This is further suggested by the observation that stimulation of adenylyl cyclase with PGE₂ or isoproterenol also inhibits changes in receptor expression. Moreover, the cAMP analogue, dibutyryl cAMP, was able to inhibit both the increase in CD11b and the decrease in L-selectin on neutrophils and eosinophils. About 45% inhibition of PAF-induced CD11b expression on neutrophils was observed at a concentration of 10⁻² M dibutyryl cAMP. This is similar to the percentage found in a study by WOLLNER *et al.* [38], in which 58% inhibition of the fMLP-induced CD11b expression on neutrophils was observed in the presence of the same concentration of dibutyryl cAMP. In contrast, BROWN *et al.* [39] did not observe an effect of dibutyryl cAMP on neutrophil CD11b expression. The reason for the different outcome of this latter study is unknown.

Inhibition by cAMP-elevating agents has been reported for several Ca²⁺-dependent granulocyte functions, including leukotriene and prostaglandin production, respiratory burst and degranulation [20, 22, 23, 25, 40]. This inhibition may be mediated by binding of cAMP to protein kinase A (PKA) and subsequent subunit dissociation. This step has been shown to uncouple heterologous receptors from phospholipase C (PLC), resulting in the inhibition of agonist-dependent increases in intracellular free Ca²⁺ concentrations [41, 42]. In the present study, PAF was used as an agonist, and it has been

found that PAF acts, at least partly, *via* the PLC pathway [43, 44]. This pathway results in a subsequent transient rise in intracellular Ca^{2+} , which can be attenuated by rolipram [24]. A rise in intracellular Ca^{2+} has also been shown to play a role in upregulation of CD11b expression [45, 46]. Thus, the inhibition of PAF-induced CD11b increase by rolipram and other cAMP-elevating agonists may be mediated by downmodulation of the capacity of PAF to activate PLC. The observation that changes in CD11b and L-selectin are concomitantly modulated by PAF and inhibited by cAMP-elevating agents suggests that both receptors are, at least partly, under control of the same Ca^{2+} -dependent transduction pathway.

A synergistic inhibition of rolipram and isoproterenol or PGE_2 was expected because of potentiation of cAMP production found in previous studies [24]. In addition, it has been shown that rolipram and isoproterenol synergistically inhibit Ca^{2+} ionophore-induced chemiluminescence, although this was dependent upon the time at which the response was measured [23]. A cumulative, instead of a synergistic, effect with respect to PAF-induced adhesion receptor expression was observed in the present study. There are various possible explanations for the lack of synergy. Firstly, a mechanism other than increase of cAMP may be involved, thereby masking synergy of rolipram with isoproterenol or PGE_2 . DERIAN *et al.* [27] have shown that rolipram and endogenously produced adenosine work in concert to affect neutrophil adhesion. Inhibition of CD11b expression by adenosine on fMLP-stimulated neutrophils has been reported [38]. As in the present study, a maximum inhibition of about 50% could be reached. It was concluded that adenosine exerted this effect *via* A2 receptors and possibly *via* activation of adenylyl cyclase. A2 receptors are also involved in inhibition of eosinophils [47]. CRONSTEIN *et al.* [48], however, have proposed that inhibition by adenosine is mediated not only by an increase in cAMP but also *via* an alternative mechanism of action. Secondly, rolipram may potentiate the cAMP production, as has been described previously [24], but the amount of intracellular cAMP may be far more than is needed for maximal inhibition. This is supported by the demonstration that rolipram and the β -adrenoceptor agonist, albuterol, exhibit synergy in elevating intracellular cAMP in human eosinophils, but do not act synergistically in suppressing respiratory burst [36]. These hypotheses could not be tested in the present study, since a mixture of different cell types were used. Isolated neutrophils and eosinophils will probably not be similar to the cells in whole blood. The cumulative increased inhibition observed in the presence of isoproterenol or PGE_2 may be of relevance *in vivo*. The concentration of prostaglandins, for instance, has been shown to be increased in bronchoalveolar lavage after instillation of ragweed allergen extracts in allergic asthmatics [49].

In conclusion, we have shown that rolipram inhibits CD11b increase and L-selectin shedding of platelet-activating factor-stimulated neutrophils and eosinophils in whole blood. With respect to CD11b expression, the inhibition by rolipram alone or in combination with isoproterenol or prostaglandin E_2 did not exceed 50%. Inhibition of shedding of L-selectin from eosinophils, but not neutrophils, was found to be 70% with a combination of low concentrations of rolipram and prostaglandin

E_2 or isoproterenol. Inhibition by type IV phosphodiesterase inhibitors of the initial rolling of eosinophils may, therefore, be a relevant subject for further investigation.

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