Rolipram Inhibits Polarization and Migration of Human T Lymphocytes

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Phosphodiesterase inhibitors possess anti-inflammatory and immunomodulatory properties and seem to have a great potential in the treatment of inflammatory skin diseases; however, an overall study on the effects of specific phosphodiesterase inhibitors, such as rolipram on the processes involved in the extravasation of lymphoid cells has not been performed. In this work we have assessed the effect of rolipram on the adhesion, polarization, and migration of normal human T lymphocytes. We found that low concentrations of rolipram were able to inhibit significantly the adhesion of T cells to the β_1 and β_2 integrin ligands vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. Rolipram also interfered with the activation of integrins, and significantly inhibited the homotypic aggregation of T lymphocytes induced by anti-β_1 and anti-α_4 integrin chain monoclonal antibodies. In addition, rolipram had a downregulatory effect on the activation of T cells, and significantly diminished the expression of the activation antigens CD69, CD25, and CD98 induced by phytohemagglutinin. Finally, this drug inhibited the polarization and transendothelial migration of T lymphocytes induced by the chemokine CXCL12 (SDF-1) and the chemotactic cytokine interleukin-15. The results indicate that rolipram, at low concentrations, exerts an important anti-inflammatory and immunomodulatory effect, and suggest that this selective phosphodiesterase inhibitor may be an effective tool for the therapy of immune-mediated diseases. ***Key words: cell adhesion/chemokines/inflammation/integrins/phosphodiesterase. J Invest Dermatol 121:81–87, 2003***

Leukocyte–endothelial cell interactions play a crucial part in different biologic phenomena, such as lymphocyte homing and inflammation. During the inflammation process, endothelial cells become activated and increase their expression of adhesion molecules, which allow their interaction with blood leukocytes. The accumulation of leukocytes into inflamed tissues occurs as a consequence of the transendothelial migration of these cells and their subsequent movement to the inflammatory foci (González-Amaro *et al*, 1998a; González-Amaro and Sánchez-Madrid, 1999). The process of leukocyte extravasation involves a cascade of adhesive events between leukocytes and endothelial cells. Initially, circulating leukocytes tether to and roll over activated endothelium, which express adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1, and E-selectin. Then, leukocytes become activated by chemokines that induce the activation of leukocyte integrins, allowing the firm adhesion of these cells to the endothelium. Finally, leukocytes migrate between the endothelial cells and move through the extracellular matrix towards the inflammatory foci (Díaz-González and Sánchez-Madrid, 1998).

In order to migrate, lymphocytes require to be activated and become polarized. During the interaction of lymphocytes with endothelial cells and extracellular matrix, chemokines induce the polarization of T lymphocytes, with the generation of two specialized compartments, the leading edge at the front of the cell, and the uropod, at the back end (del Pozo *et al*, 1998). The chemokine receptors involved in the detection of chemoattractant gradients are concentrated at the leading edge of the cell, whereas adhesion molecules such as ICAM-1, ICAM-3, CD44, and CD43 are redistributed to the uropod (del Pozo *et al*, 1998). It has been shown that the uropod is involved in the recruitment of bystander leukocytes through LFA-1/ICAM-1-dependent cell–cell interactions (del Pozo *et al*, 1997). Chemokines play a key part in the activation and chemotaxis of leukocytes. Lymphocyte migration is induced by different chemokines such as CCL5 (RANTES), CCL3 (MIP-1α), and CCL2 (MCP-1) (DeVries *et al*, 1995; Moser and Loetscher, 2001). In addition, the stromal cell-derived factor 1 (SDF-1 or CXCL12) is involved in the chemotaxis of lymphocytes towards inflamed tissues as well as in the migration of these cells to lymph nodes (Nanki *et al*, 2000; Zlotnik and Yoshie, 2000; Phillips and Ager, 2002). Other chemotactic factors, such as interleukin (IL)-15 have been shown to induce lymphocyte migration and cell polarization (Wilkinson and Liew, 1995).

Cyclic adenosine monophosphate (cAMP) has been shown to modulate inflammatory and immune processes (Bourne *et al*, 1974). Phosphodiesterase (PDE) inhibitors increase intracellular

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Abbreviations: cAMP, cyclic adenosine monophosphate; HUVEC, human umbilical vein endothelial cells; PDE, phosphodiesterase; PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor alpha.

cAMP by blocking cAMP conversion to 5′AMP. Although several PDE are present in immune cells (e.g., PDE3, PDE4, and, to a lesser extent, PDE7), PDE4 seems to be the major contributor to cAMP hydrolysis in lymphocytes, monocytes, polymorphonuclear leukocytes, basophils, and eosinophils (Torphy, 1998). Rolipram is a specific inhibitor of PDE4, an enzyme that exclusively hydrolyzes cAMP (Bolger *et al*, 1993). Rolipram exerts complex immunomodulatory effects. It has been shown that this drug has a beneficial effect in experimental autoimmune encephalomyelitis (Martínez *et al*, 1999), collagen-induced arthritis (Ross *et al*, 1997) as well as in airways inflammation and hyperresponsiveness (Kanehiro *et al*, 2001). In addition, it has been reported that rolipram regulates cytokine synthesis through inhibition of nuclear factor-κB and NFAT transcription factors (Jiménez *et al*, 2001). On the other hand, rolipram has been described to inhibit the adhesion of neutrophils to endothelial cells (Derian *et al*, 1995) as well as the allergen-induced migration of eosinophils (Silva *et al*, 2001). Furthermore, the staphylococcal enterotoxin B-induced expression of the T cell skin homing receptor CLA is inhibited by rolipram (Santamaria *et al*, 1999). In addition, several effects of rolipram on endothelial cells have been described (Blease *et al*, 1998; Folcik *et al*, 1999).

The potential role of PDE inhibitors in the therapy of immune-mediated skin diseases has been previously explored. Pentoxifylline, a nonspecific PDE inhibitor, seems to be of value in the therapy of inflammatory conditions such as graft versus host disease, Schamberg's disease, leprosy reaction, and allergic hypersensitivity reactions (Samlaska and Winfield, 1994; Kano *et al*, 1997; Moraes *et al*, 2000). *In vitro*, this drug has different downregulatory effects on leukocytes, including tumor necrosis factor (TNF)-α synthesis, cell adhesion and migration (Wang *et al*, 1997; González-Amaro *et al*, 1998b; Domínguez-Jiménez *et al*, 2002). The levels of pentoxifylline required to exert these effects, however, are high and it is not clear that these concentrations are reached *in vivo*. Therefore, it is of interest to make an overall assessment of the *in vitro* anti-inflammatory properties of other PDE inhibitors that have higher potency and a selective effect on the PDE expressed by lymphocytes. The aim of this work was to explore the effect of the PDE4-specific inhibitor rolipram on key phenomena involved in inflammation. We found that this drug exerts, at low concentrations, a downregulatory effect on lymphocyte activation, polarization, adhesion, and transendothelial migration. These data strongly support the anti-inflammatory and immunomodulatory potential of this drug.

MATERIALS AND METHODS

Cells Peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Hypaque gradient centrifugation. T lymphoblasts were obtained by removing non-T cells from the peripheral blood mononuclear cells by nylon wool adherence, and stimulating with 5 μg per mL phytohemagglutinin (PHA-P; Sigma, St Louis, Missouri) for 48 h. Then, cells were washed and cultured in RPMI 1640 medium (Whittaker, Walkersville, Maryland) containing 10% fetal calf serum and 25 U per mL IL-2 (R&D Systems, Minneapolis, Minnesota). T lymphoblasts cultured for 2 to 4 d were typically used in all experiments. Freshly isolated T lymphocytes were obtained from peripheral blood mononuclear cells by rosetting with sheep red blood cells. Cell viability was assessed by trypan blue dye exclusion and the percent of apoptotic cells was determined by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) and flow cytometry using the APO-DIRECT staining kit (Phoenix Flow Systems, San Diego, California), according to the manufacturer's instructions.

Antibodies, cytokines, and reagents Rolipram was obtained from Sigma and was used from 10^{-7} to 10^{-3} M. The T3b anti-CD3, TP1/55 anti-CD69, TP1/6.2 anti-CD25, TP1/24 anti-ICAM-3, FG1/8 anti-CD98, and the Lia1/2 anti-β_1, and HP1/7 anti-α_4 integrin chains as well as the blocking TP1/40 anti-CD11a and HP1/2 anti-α_4 integrin chain monoclonal antibodies (MoAb) were kindly provided by Dr F. Sánchez-Madrid, Hospital de La Princesa (Madrid, Spain), and have been previously described (Cebrián *et al*, 1988; Campanero *et al*, 1992, 1993; Melero *et al*, 1993). The TS2/16 is an activating anti-β_1 integrin MoAb that has been previously described (Hemler *et al*, 1984). The HUTS-21 MoAb is specific for an activation epitope of β_1 integrins (Luque *et al*, 1996), and was a generous gift of Dr C. Cabañas, Consejo Superior de Investigaciones Científicas (Madrid, Spain). Chimeric ICAM-1-Fc, and VCAM-1–4D-Fc, consisting of the total extracellular domains fused to IgG Fc fragment, were obtained as described (Berendt *et al*, 1992). Phorbol myristate acetate (PMA) and TNF-α were purchased from Sigma, and IL-15 from Immunex Corp. (Seattle, Washington). CXCL12 (SDF-1) was obtained from PeproTech (Rocky Hill, New Jersey).

Cell adhesion assays Cell adhesion assays were performed as previously described (Campanero *et al*, 1993; Luque *et al*, 1996). Briefly, 96-well microtiter EIA II-Linbro plates (Costar, Cambridge, Massachusetts) were coated with recombinant chimeric ICAM-1-Fc (10 μg per mL), or VCAM-1-4D-Fc (5 μg per mL), and nonspecific binding sites were saturated with 1% human serum albumin. Then, plates were washed three times with PBS and 1×10^5 T lymphoblasts or freshly isolated T cells, previously incubated in the presence or absence of 10^{-5} M rolipram and then treated or not with PMA, were added to each well. After centrifugation at $10 \times g$ for 5 min, plates were incubated at 37°C for 20 min. To quantify cell attachment, the plates were washed thrice with RPMI 1640, and cells were fixed with methanol/acetone (1:1), and stained with violet crystal 0.5%. Violet crystal was then extracted with sodium citrate 0.1 M, pH 4.2/ethanol, and absorbance at 540 nm was measured in a Multiskan EX ELISA reader (Labsystems Oy, Helsinki, Finland). All assays were run in duplicate, and results were expressed as the percentage of bound cells. The absorbance of 1×10^5 cells, which were fixed and stained without previous washing, was considered as 100% of cell adhesion. Specificity of cell adhesion was corroborated using blocking MoAb (TP1/40 anti-CD11a and HP1/2 anti-α_4 integrin chains) or bovine serum albumin as substratum.

Cell aggregation assays Homotypic cell aggregation assays were performed as previously described (Campanero *et al*, 1993). Briefly, T lymphocytes (1×10^5) pretreated or not with 10^{-5} M rolipram for 24 h, were incubated in flat-bottom 96-well microtiter plates (Costar) in a final volume of 100 μL of complete RPMI 1640 medium. Then, the pro-aggregatory Lia1/2 anti-β_1 and the HP1/7 anti-α_4 integrin chain MoAb were added at a concentration of 5 μg per mL, and cells were allowed to settle at 37°C for 3 h. Cell aggregation was determined by direct visualization of the plate with an inverted microscope and counting the free cells of at least five randomly chosen fields. All assays were conducted by duplicate and results were expressed as percentage of aggregated cells, which was obtained by the following formula: percent aggregation = 100 × (1–[number of free cells])/(total number of cells).

Flow cytometry analysis T lymphocytes incubated with medium alone, PMA or the activating anti-β_1 integrin MoAb TS2/16 in the absence or presence of 10^{-5} M rolipram for 24 h, were washed and incubated with the biotinylated HUTS-21 MoAb, following by washing and labeling with fluorescein isothiocyanate–avidin. The fluorescence produced by the isotype-matched myeloma P3X63 supernatant was considered as background. Cells were analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, California), and results were expressed as the mean fluorescence intensity. For the analysis of the expression of activation antigens, mono-nuclear cells were stimulated with 5 μg PHA per mL for 48 h in the absence or presence or 10^{-5} M rolipram. Then, cells were washed and incubated with the T3b anti-CD3, TP1/55 anti-CD69, TP1/6.2 anti-CD25, or the FG1/8 anti-CD98 MoAb, followed by washing and labeling with an fluorescein isothiocyanate-labeled rabbit anti-mouse IgG. Results were expressed as the percentage of positive cells.

Cell polarization assays Lymphocyte polarization assays were performed as described (Nieto *et al*, 1997). Briefly, 2×10^6 T cells or T lymphoblasts pretreated or not with different concentrations of rolipram, were allowed to adhere to coverslips precoated with fibronectin for 1 h at 37°C, 5% CO_2 in 24-well plates (Costar, Cambridge, MA). Then, cells were incubated with CXCL12 (100 ng per mL), IL-15 (10 ng per mL), or medium alone for 30 min and fixed. Finally, cells were immunostained with the TP1/24 anti-ICAM-3 MoAb followed by a fluorescein isothiocyanate-tagged rabbit anti-mouse IgG (Coulter Corp., Hialeah, Florida), and the proportion of uropod-bearing cells was calculated using a Nikon Labophot-2 epifluorescence microscope (Tokyo, Japan). Microphotographs were obtained with a Nikon 850 Coolpix digital camera.

Lymphocyte-human umbilical vein endothelial cells (HUVEC) adhesion assay HUVEC, obtained as described (Jaffe *et al*, 1973), were grown to confluence on gelatin-coated coverslips in 24-well plates. Peripheral blood T lymphocytes were pretreated or not with different concentrations of rolipram for 8, 12, and 24 h. Then, cells were loaded with the fluorescent probe BCECF-AM (Molecular Probes, Eugene, Oregon), washed, resuspended at 2×10^6 cells per mL in M199 medium with 0.05% human serum albumin, and allowed to adhere to the HUVEC monolayer (500 μL of lymphocyte suspension per well) in the presence of CXCL12 (100 ng per mL) or medium alone for 5 or 10 min at 37°C and 5% CO_2. Then, coverslips were washed, fixed, and visualized in a Nikon Labophot-2 microscope. The number of lymphocytes (fluorescent cells) that were adhered to endothelium was counted in 10 random fields (63 × oil-immersion objective), and results were expressed as the total number of cells registered.

Transendothelial migration assays HUVEC were grown to confluence on Transwell inserts precoated with gelatin (Sigma) and activated or not with TNF-α (20 ng per mL) for 6 h. T lymphocytes (1×10^6), pretreated or not for 12 h with different concentrations of rolipram, were added to the upper chamber, and 600 μL of medium alone or with CXCL12 (SDF-1, 50 ng per mL) were added to the lower compartment. Cells were incubated for 16 h at 37°C, and migrated cells were recovered from the lower chamber, mixed with TruCount beads (Becton-Dickinson) and counted during 1.0 min using a FACSCalibur flow cytometer. Results were expressed as the number of migrated cells. In additional experiments, T cells (5×10^6) pretreated or not with rolipram were labeled with 100 μCi of $Na^{51}CrO_4$ during 1 h, washed and incubated for an additional 60 min in an excess of culture medium. Then, labeled cells were employed for migration assays as described above. At the end of incubation, the radioactivity (cpm) in the upper and the bottom chambers was determined in a gamma spectrometer (Packard, Ames, Iowa) and results were expressed as the migration index.

RESULTS

Rolipram inhibits the adhesion of T cells to β_1 and β_2 integrin ligands in a concentration- and time-dependent manner One of the events required for leukocyte migration into inflamed tissues involves the integrin-mediated adhesion of lymphocytes to endothelial cells (González-Amaro and Sánchez-Madrid, 1999). We investigated the effect of rolipram on T lymphocyte adhesion to the β_1 and β_2 integrin ligands VCAM-1 and ICAM-1. As shown in **Fig 1**(***A,B***) basal adhesion of T lymphoblasts to ICAM-1 and VCAM-1 was significantly inhibited by rolipram at 10^{-5} M ($p < 0.05$ in both cases, Mann–Whitney U test). In addition, the adhesion of T lymphoblasts to ICAM-1 and VCAM-1 induced by the protein kinase C activator PMA was also significantly inhibited by rolipram ($p < 0.05$). Similar results were observed when experiments were conduced using freshly isolated T lymphocytes and ICAM-1-coated plates (**Fig 1*C***). As expected, control blocking anti-CD11a (TP1/40, **Fig 1*A,C***) and anti-α_4 integrin chain (HP1/2, **Fig 1*B***) MoAb inhibited the adhesion of T cells to ICAM-1 and VCAM-1, respectively. Next, we performed concentration- and time-response experiments. We found that rolipram decreased the PMA-induced adhesion of T cells to VCAM-1 and ICAM-1, in a concentration-dependent fashion, with a significant effect at 10^{-6} to 10^{-5} M for both T lymphocytes and T lymphoblasts (**Fig 2*A***). On the other hand, kinetics experiments revealed that the effect of rolipram on PMA-stimulated T cell adhesion to VCAM-1 and ICAM-1 was evident as early as after 4 h, with a maximal effect after 12 h (**Fig 2*B***). The observed effect of rolipram was not related to cell death and T lymphocyte viability remained unaffected at all concentrations of rolipram tested (10^{-7}–10^{-3} M) and after 24 h of incubation (**Fig 2*C***). Likewise, no significant effect of rolipram on the apoptosis of T cells or T lymphoblasts was observed (**Fig 2*C***). Similar results were obtained at 6 and 12 h of incubation with rolipram (data not shown).

Effect of rolipram on β_1 integrin activation and β_1 integrin-induced homotypic aggregation of T lymphocytes The inhibitory effect of rolipram on the adhesion of T lymphocytes to integrin ligands prompted us to explore whether the activation state of integrins was affected by this drug. By using the HUTS-21 MoAb, which specifically recognizes an activation

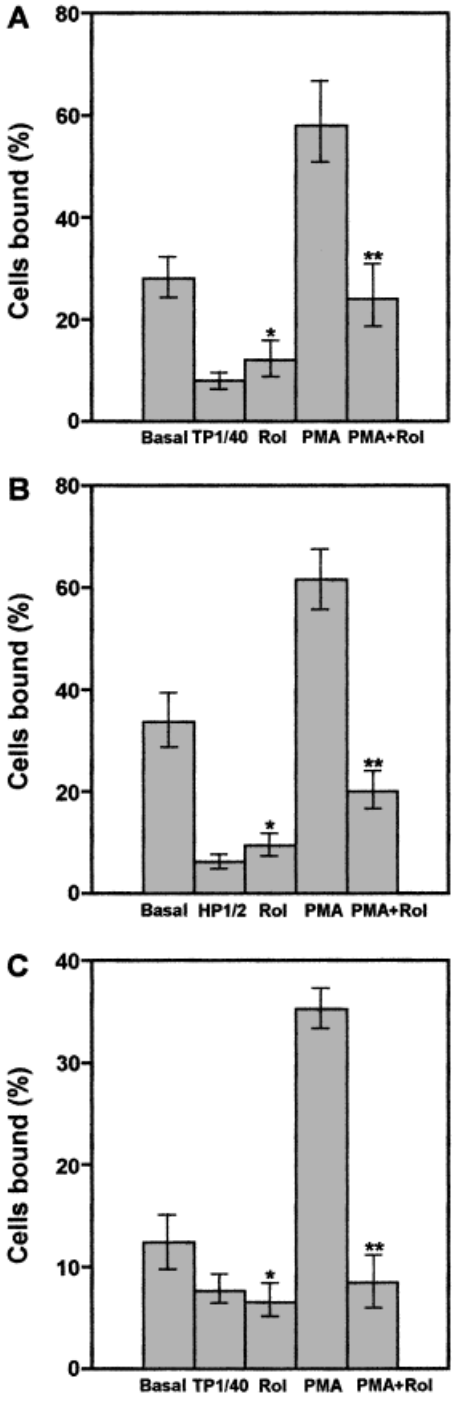

Figure 1. Rolipram inhibits the adhesion of T cells to β_1 and β_2 integrin ligands. T lymphoblasts (*A,B*) or T cells (*C*) were incubated in the presence or absence of rolipram (10^{-5} M) for 24 h, then stimulated with or without PMA (20 ng per mL) and allowed to adhere to recombinant ICAM-1-coated plates (*A,C*) or VCAM-1-coated plates (*B*). Blocking anti-CD11a (TP1/40) (*A,C*) and anti-VLA-4 (HP1/2) (*B*) were added to inhibit cell binding to ICAM-1 (*A,C*) and VCAM-1 (*B*), respectively. Finally, the percentage of cell adhesion was determined by crystal violet staining, as described in *Materials and Methods*. Values of cell adhesion correspond to the arithmetic mean ± SD of five independent experiments. *$p < 0.05$ compared with basal; **$p < 0.05$ compared with PMA.

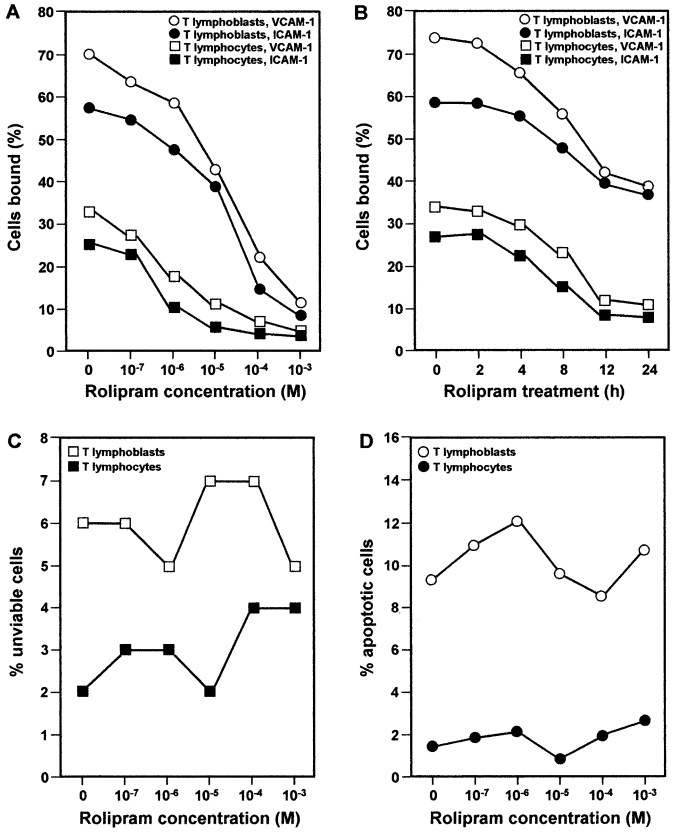

Figure 2. Concentration- and time-dependent analyses of effect of rolipram on adhesion of T cells to ICAM-1 and VCAM-1 and on the induction of cell death. T cells pretreated with rolipram at the indicated doses for 24 h (*A*), or for the specified periods of time at 10^{-5} M (*B*) were stimulated with PMA and then were allowed to adhere to recombinant ICAM-1, or VCAM-1-coated plates. Then, the percentage of cell adhesion was determined by crystal violet staining, as described in *Materials and Methods*. Data of a representative experiment out of three are shown. (*C*,*D*) Lack of significant effect of rolipram on T cell viability and apoptosis. T lymphocytes were treated as in (*A*) and cell viability was assessed by trypan blue dye exclusion (*C*), and apoptosis by TUNEL and flow cytometry. Data of a representative experiment of four are shown.

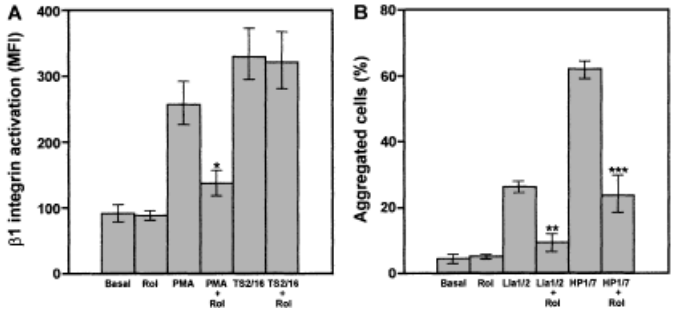

Figure 3. Effect of rolipram on β_1 integrin activation and β_1 integrin-induced homotypic aggregation of T cells. (*A*) T lymphocytes were incubated in the presence or absence of 10^{-5} M rolipram and then stimulated with PMA (20 ng per mL) or the activating MoAb TS2/16. Thereafter, β_1 integrin activation was assessed by flow cytometry using the HUTS-21 MoAb. Data correspond to the arithmetic mean ± SD of mean fluorescence intensity of five independent experiments. (*B*) T lymphocytes were incubated with the pro-aggregatory Lia1/2 anti-β_1 integrins, and HP1/7 anti-α_4 integrin chain MoAb with or without rolipram 10^{-5} M. After an incubation period of 3 h, the percentage of aggregated cells was determined as described in *Materials and Methods*. Data correspond to the arithmetic mean ± SD of the percentage of aggregated cells of five independent experiments. *$p < 0.05$ compared with PMA; **$p < 0.05$ compared with Lia1/2 alone; ***$p < 0.05$ compared with HP1/7 alone.

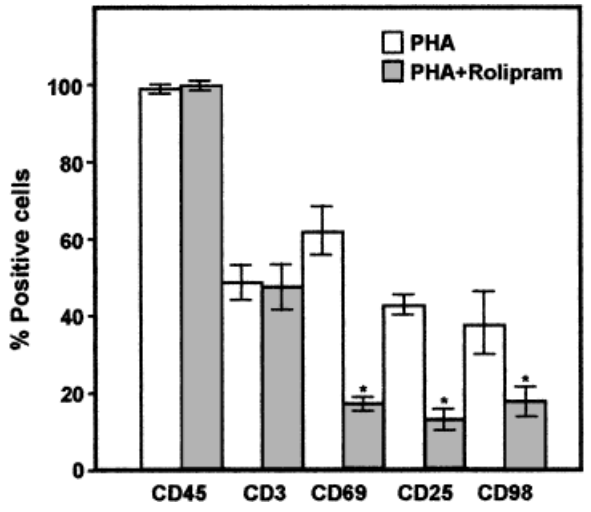

Figure 4. Rolipram inhibits the expression of CD69, CD25, and CD98 activation antigens by PHA-stimulated lymphocytes. Mononuclear cells were stimulated with 5 μg PHA per mL for 48 h with or without 10^{-5} M rolipram. Then, the expression of CD69, CD25, and CD98 activation antigens, and CD3 and CD45 was assessed by flow cytometry, as described in *Materials and Methods*. Data correspond to the arithmetic mean ± SD of the percentage of positive cells of five independent experiments. *$p < 0.05$ compared with PHA alone.

neoepitope on β_1 integrins, we observed that rolipram at 10^{-5} M significantly inhibited PMA-induced activation of β_1 integrins ($p < 0.05$), but had no effect on the basal levels of integrin activation (**Fig 3*A***). In contrast, the activation of these adhesion receptors induced by the activating TS2/16 MoAb was no affected by rolipram (**Fig 3*A***). As in the cell adhesion assays, the maximal effect of rolipram on integrin activation was observed at 12 to 24 h (data not shown).

To analyze further the regulatory role of rolipram on β_1 integrin-mediated adhesion of T lymphocytes, we explored the effect of this drug on the homotypic aggregation of T cells, a process that involves both cell adhesion and cell migration (Serrador *et al*, 1998). As shown in **Fig 3(*B*)** rolipram at 10^{-5} M for 12 h significantly inhibited the homotypic aggregation of T lymphocytes induced by the Lia1/2 anti-β_1 integrins, and the HP1/7 anti-α_4 integrin chain MoAb ($p < 0.05$).

Effect of rolipram on the activation of T cells As activation of T cells is a key event required for the expression and activation of adhesion molecules that mediate the extravasation of lymphocytes (Díaz-González and Sánchez-Madrid, 1998), we evaluated the effect of rolipram on the PHA-induced expression of the activation antigens CD25, CD69, and CD98. We found that rolipram significantly inhibited the expression of all activation antigens studied on the surface of PHA-stimulated T lymphocytes ($p < 0.05$ in all cases) (**Fig 4**). As expected, rolipram had no effect on CD3 or CD45 levels (**Fig 4**), indicating that this drug selectively inhibits the expression of cell surface markers that are upregulated during T cell activation.

Rolipram inhibits T lymphocyte polarization induced by IL-15 and CXCL12 In order to assess T lymphocyte polarization, cells were immunostained for ICAM-3, a molecule that redistributes to the uropod, and the proportion of uropod-bearing cells was calculated. Rolipram significantly reduced the

polarization of peripheral blood T lymphocytes induced by the chemokine CXCL12, and the chemotactic cytokine IL-15 ($p < 0.05$) (**Fig 5**). This effect was concentration-dependent, with a significant inhibition at 10^{-6} to 10^{-5} M. Kinetics experiments with 10^{-5} M rolipram revealed that its inhibitory effect on T cell polarization was evident after 8 h of incubation, with a maximal effect at 24 h (data not shown). In contrast, rolipram had no effect on the spontaneous polarization of T lymphocytes (**Fig 5*A***). This drug also significantly inhibited IL-15- and CXCL12-induced polarization of T lymphoblasts as well as the baseline polarization of these cells ($p < 0.05$) (**Fig 5*B***).

Rolipram inhibits transendothelial migration As we found that rolipram inhibited lymphocyte polarization in response to CXCL12 and IL-15, we decided to assess the effect of this drug on the adhesion of T lymphocytes to endothelial cells and their transendothelial migration. As expected, rolipram inhibited adhesion of both resting and CXCL12-stimulated T lymphocytes to endothelial cells in a concentration-dependent fashion (**Fig 6*A***). This effect was observed after 8, 12, or 24 h of rolipram treatment and at 5 or 10 min of T cell/HUVEC interaction (**Fig 6*A*** and data not shown). In addition, the basal (**Fig 6*B***) as well as CXCL12-induced (**Fig 6*C***) transendothelial migration of T lymphocytes was significantly inhibited by rolipram ($p < 0.05$). This effect was observed when both resting and TNF-α-activated endothelial cells were used (**Fig 6*B,C***). Similar results were obtained when migration assays were performed with ^{51}Cr-labeled T cells and the results expressed as the migration index (**Fig 6*D***). Under such experimental conditions, however, a more evident effect of CXCL12 on T cell chemotaxis was observed.

All these experiments were performed at 12 h of incubation with rolipram, and additional experiments showed that this drug had a similar effect at 24 h (data not shown). As in the case of rolipram alone, the different treatments employed did not significantly affect the viability of the migrated cells (data not shown).

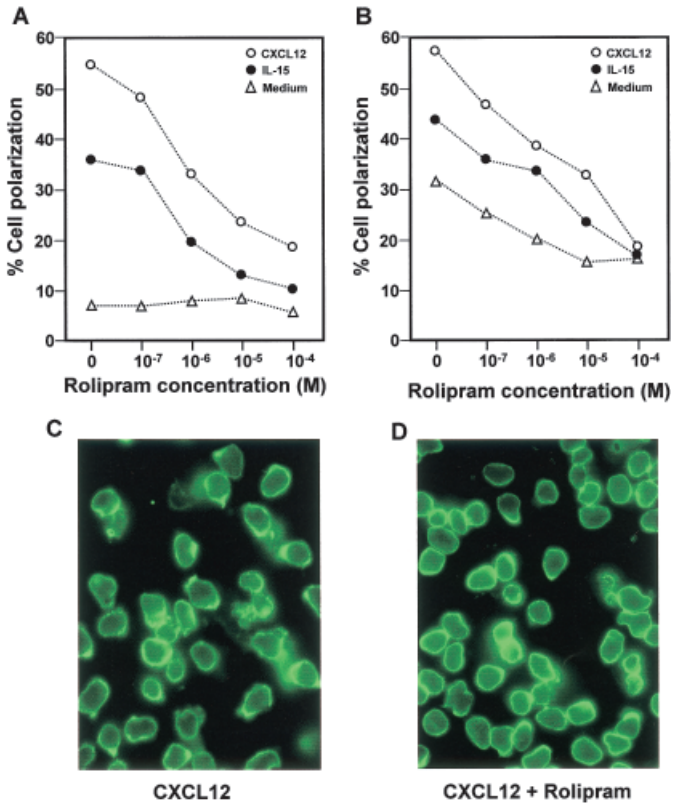

Figure 5. Rolipram inhibits T lymphocyte polarization induced by IL-15 and CXCL12. Peripheral blood T lymphocytes (*A*) or T lymphoblasts (*B*), pretreated with the indicated concentrations of rolipram, were allowed to adhere to fibronectin 80-coated coverslips and then stimulated with CXCL12 (100 ng per mL), IL-15 (10 ng per mL), or medium alone. Thereafter, cell polarization was assessed by ICAM-3 immunostaining, as described in *Materials and Methods*. Data of a representative experiment of five performed are shown. Images from a representative experiment of CXCL12-induced polarization of T lymphocytes in the presence (*D*) or absence (*C*) of rolipram are also shown. Note the scarcity of lymphocytes with ICAM-3 redistributed to a cellular pole (polarized cells) in (*D*) compared with (*C*).

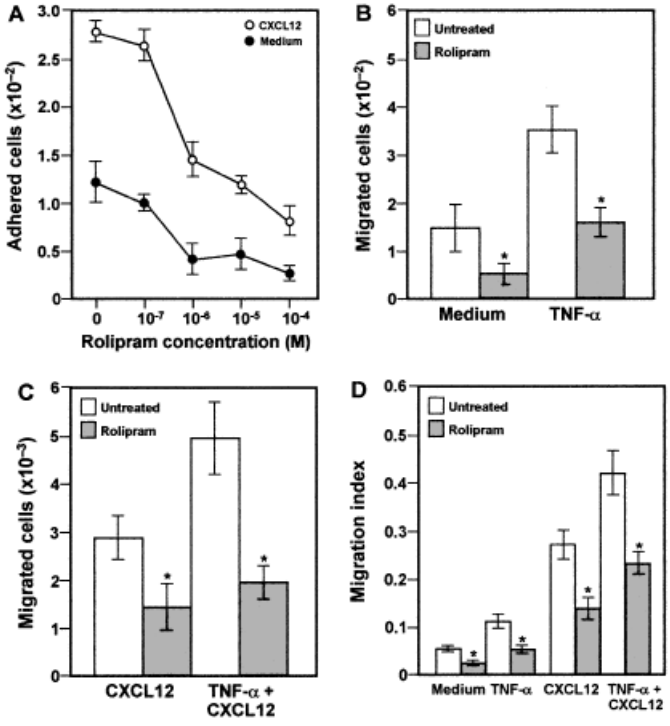

Figure 6. Rolipram interferes with the adhesion of T cells to endothelial cells and their transendothelial migration. (*A*) BCECF-loaded T lymphocytes pretreated with the indicated concentrations of rolipram for 24 h were allowed to adhere to resting HUVEC in the presence or not of CXCL12 (100 ng per mL) for 10 min. Thereafter, cell adhesion was quantified by fluorescence microscopy, as described in *Materials and Methods*. (*B,C*). T lymphocytes incubated in the presence or absence of 10^{-5} M rolipram for 24 h were poured in the upper compartment of Transwell chemotaxis chambers. The upper side of the Transwell membrane was precoated with a confluent monolayer of HUVEC that was treated with or without 20 ng per mL TNF-α for 4 h. Basal (*B*) and CXCL12-induced (*C*) migration of T lymphocytes was quantified by flow cytometry as described in *Materials and Methods*. Data correspond to the arithmetic mean ± SD of adhered cells (*A*) or migrated cells (*B,C*) of five independent experiments. (*D*) Migration assays were performed as in (*B*) and (*C*), but T cells were labeled with $Na^{51}CrO_4$ and results were expressed as the migration index. Data correspond to the arithmetic mean ± SD of migration index of four independent experiments. *$p < 0.05$ compared with untreated cells.

DISCUSSION

There is accumulating evidence that PDE inhibitors represent an interesting group of anti-inflammatory and immunomodulatory agents with great potential in the treatment of immune-mediated skin diseases (Bruynzeel *et al*, 1998). In this regard, it has been described that the nonspecific PDE inhibitor pentoxifylline has

a beneficial effect in allergic hypersensitivity reactions, graft versus host disease, leprosy reaction, and Schamberg's disease (Samlaska and Winfield, 1994; Kano *et al*, 1997; Moraes *et al*, 2000). It is likely, however, that the usual doses of this drug are not always sufficient to obtain the tissue concentrations necessary to exert the different anti-inflammatory effects described *in vitro* for this PDE inhibitor (Wang *et al*, 1997; González-Amaro *et al*, 1998b; Domínguez-Jiménez *et al*, 2002). Therefore, we decided to assess *in vitro* the anti-inflammatory properties of rolipram, a potent and selective PDE inhibitor. Interestingly, we have found that low concentrations of this drug are able to inhibit different key immune phenomena, including lymphocyte activation, polarization, adhesion, and transendothelial migration. As we have previously found for pentoxifylline (González-Amaro *et al*, 1998b), the effect of rolipram was not related to the induction of accidental (necrosis) or programmed cell death (apoptosis).

Integrins, mainly those from β_2 subfamily and VLA-4, play an essential part in extravasation of leukocytes towards inflammatory foci (González-Amaro *et al*, 1998a; González-Amaro and Sánchez-Madrid, 1999). It has been described that the LFA-1/ICAM-1 adhesion pathway is involved in cell–cell interactions and the firm adhesion of leukocytes to endothelial cells (Postigo *et al*, 1993). In addition, the interaction between VLA-4 and VCAM-1 is essential for the rolling of lymphoid cells on activated endothelium, both *in vivo* and *in vitro* (Johnston *et al*, 1996). We found that rolipram inhibits the adhesion of T cells to the β_1 and β_2 integrin ligands VCAM-1 and ICAM-1. A significant inhibition was seen at 10^{-6} to 10^{-5} M for both T lymphocytes and T lymphoblasts. These concentrations are significantly lower than those required for pentoxifylline to inhibit the adhesion of T cells (10^{-4}–10^{-3} M) (González-Amaro *et al*, 1998b). Accordingly, it has been described that 10^{-5} M rolipram inhibits the adhesion of neutrophils to endothelial cells *in vitro* (Derian *et al*, 1995), and the migration of eosinophils induced by antigenic challenge *in vivo* (Silva *et al*, 2001).

As lymphocyte adhesiveness is closely related to the activation state of these cells, we studied the effect of rolipram on the expression of activation markers on the surface of T cells. We found that the PHA-induced expression of CD69, CD25, and CD98 on the surface of T lymphocytes was significantly reduced by rolipram. In accordance, Kasyapa *et al* (1999) found a reduced expression of CD69 on the surface of T cells pretreated with rolipram and stimulated with IL-15. Furthermore, our data are in agreement with the study of Bielekova *et al* (2000) who reported that rolipram downregulates the expression of class II major histocompatibility complex antigens and CD80 in monocytes and lymphocytes upon activation with lipopolysaccharide or PHA. On the other hand, we have found that rolipram inhibits the activation of β_1 integrins induced by PMA, but not by an activating MoAb, which directly induces conformational changes of integrins that increase the affinity for their ligands. These data suggest that rolipram affects an intracellular signaling pathway that involves protein kinase C and that results in integrin activation. In this regard, it has been described that protein kinase C activation is required for the expression of CD69 (Cebrián *et al*, 1989), which is inhibited by rolipram.

Lymphocyte activation induces cell polarization with the subsequent redistribution of adhesion receptors to the uropod, a phenomenon that is critical for lymphocyte chemotaxis and recruitment to inflammatory foci (del Pozo *et al*, 1996). Thus, the inhibitory effect of rolipram on the transendothelial migration of T lymphocytes in response to CXCL12 (SDF-1) is in accordance with its downregulatory effect on lymphocyte polarization. It is of interest that rolipram affects the cell polarization induced by two distinct stimuli, a chemokine and IL-15, that bind to very different receptors. These data suggest that rolipram, as pentoxifylline (Domínguez-Jiménez *et al*, 2002), affects an intracellular signaling pathway that is common to cell membrane receptors involved in the induction of lymphocyte polarization. In agreement with this point, Hidi *et al* (2000) reported that rolipram is able to inhibit chemotaxis of T lymphocytes induced by two different chemoattractants, IL-8 and platelet activation factor. It is worth mentioning that CXCL12 is expressed by dendritic cells and endothelium in human skin and may play an important part in the recruitment of lymphocytes in inflammatory skin diseases (Pablos *et al*, 1999). Thus, the down-modulatory effect of rolipram on lymphocyte migration may be beneficial for patients suffering from skin diseases in which lymphocyte infiltration is a central feature, such as psoriasis.

The phenomenon of intercellular adhesion that is observed when leukocytes are incubated *in vitro* with MoAb that bind to cell surface receptors is known as homotypic aggregation. It has been proposed that the ligation of these molecules on the surface of leukocytes triggers a cascade of intracellular signals that induces cell migration and increase the affinity of leukocyte integrins for their ligands, leading to the process of intercellular adhesion. These phenomena are critical for the development of inflammatory and immune responses and play an important part in numerous biologic processes, both normal and pathologic. Interestingly, we have found that rolipram significantly reduces the homotypic aggregation of T lymphocytes induced through β_1 integrins. It is very likely that this effect is consequence of the inhibition by rolipram of lymphocyte chemotaxis and adhesion. In addition, it is very feasible that this effect significantly contributes to the anti-inflammatory activity of this drug.

In this work we have found that rolipram inhibits different functions of T lymphocytes at concentrations 100 times lower than those of pentoxifylline, a nonselective PDE inhibitor (González-Amaro *et al*, 1998b; Domínguez-Jiménez *et al*, 2002). In agreement, Semmler *et al* (1993) reported that rolipram is 500-fold more potent than pentoxifylline in the suppression of TNF-α synthesis by human mononuclear cells induced by lipopolysaccharide. In addition, when a combination of a PDE inhibitor and methylprednisolone was tested, the percentage of inhibition of mitogen-induced lymphocyte proliferation achieved with 10^{-5} M rolipram was significantly greater than that obtained with 10^{-4} M pentoxifylline (Briggs *et al*, 1999). Thus, there are different data that clearly indicate the high potency of rolipram on the inhibition of different key functions of immune cells.

In summary, our data indicate that the type 4-specific PDE inhibitor rolipram exerts, at low concentrations, important anti-inflammatory and immunomodulatory effects. Thus, this drug as well as additional novel PDE inhibitors may be an important tool for the treatment of diseases in which T cells are involved. The clinical potential of rolipram in the treatment of immune-mediated skin diseases is expected to become a fruitful area of research. The development of a formula that allows the topical application of this drug could be useful for the treatment of skin diseases.

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