Homeostatic Intracellular-Free Ca^{2+} Is Permissive for Rap1-Mediated Constitutive Activation of α_4 Integrins on Eosinophils[1]

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Although much progress has been made in understanding the molecular mechanisms underlying agonist-induced "inside-out" activation of integrins, little is known about how basal levels of integrin function are maintained. This is particularly important for nonactivated eosinophils, where intermediate activation of $\alpha_4\beta_1$ integrin supports recruitment to endothelial cells under flow conditions. Depletion of intracellular Ca^{2+} and pharmacological inhibition of phospholipase C (but not other intracellular signaling molecules, including PI3K, ERK1/2, p38 MAPK, and tyrosine kinase activity) abrogated basal α_4 integrin activity in nonactivated eosinophils. Basal α_4 integrin activation was associated with activation of the small GTPase Rap1, a known regulator of agonist-induced integrin function. Basal Rap activation was dependent upon phospholipase C, but not intracellular Ca^{2+}. However, depletion of intracellular Ca^{2+} in $CD34^+$ hematopoietic progenitor cells abolished RapV12-mediated induction of α_4 integrin activity. Thus, residual Rap activity or constitutively active Rap activity in Ca^{2+}-depleted cells is not sufficient to induce α_4 integrin activation. These data suggest that activation of functional α_4 integrin activity in resting eosinophils is mediated by Rap1 provided that the intracellular-free Ca^{2+} is at a normal homeostatic concentration. ***The Journal of Immunology,*** **2008, 180: 5512–5519.**

Eosinophils are associated with allergic diseases such as allergic rhinitis and allergic asthma (1). These cells accumulate in the affected tissues and contribute to the chronic inflammatory reaction which is characteristic of these allergic diseases (2). To migrate from the blood to the inflamed tissues, eosinophils use a specific set of adhesion and chemokine receptors (reviewed in Ref. 3). Rolling, activation, firm adhesion, polarization, and extravasation are sequentially defined steps required for leukocyte migration out of the blood into tissue. In general, rolling interactions are mediated by selectins binding to mucin-like structures bearing specific carbohydrate moieties. Subsequently, firm adhesion is initiated by activation of the cells through chemoattractants which signal via G protein-coupled receptors. These processes result in different states of integrin activation depending on the chemoattractant present (4). Activated integrins bind to vascular adhesion molecules of the Ig superfamily which eventually results in firm adhesion. Finally, the leukocytes pass through the endothelium by either para- or transcellular processes (5).

Integrins are heterodimers that consist of an α- and a β-chain that are noncovalently linked. These integrins are under "inside-out" control: intracellular signals that are induced by inflammatory stimuli (e.g., chemokines) integrate at the intracellular tail of the integrin resulting in an active integrin receptor (reviewed in Ref. 6). Subsequently, the functionally active integrin is able to bind ligand. Both changes in conformation (affinity) as well as in clustering (valency) contribute to $\alpha_4\beta_1$ integrin activity (7, 8). Recently, it was shown that an Ab recognizing activated β_1 integrins on peripheral blood eosinophils correlated with disease severity in asthma (9). An important characteristic of α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) on lymphocytes, monocytes, as well as on eosinophils, is that they can pre-exist as adhesion receptors with an intermediate ligand-binding capacity, without the apparent need for cellular activation (10–13). Indeed, we and others have previously shown that α_4 integrins on resting, nonactivated eosinophils contribute significantly to eosinophil recruitment to activated endothelial cells (13, 14). Given the importance of α_4 integrins for recruitment of leukocytes to inflammatory sites in vivo, it is therefore important to elucidate the inside-out control mechanisms that control the intermediate and fully active conformations of α_4-containing integrins (15). Intracellular signaling pathways that maintain the basal intermediate activity of α_4 integrins have not been extensively studied. For T cells, it has been shown that the *src* kinase $p56^{lck}$ is involved (16), and in line with these findings it was demonstrated that basal α_4 integrin activity in primary basophils was inhibited by the PP1 *src* kinase inhibitor (17). For eosinophils, the role of tyrosine kinases has also been demonstrated since Matsumoto et al. (18) showed that basal eosinophil binding to VCAM-1 was inhibited by tyrphostin B46. However, in another study, the tyrosine kinase genistein did not affect α_4 integrin activity (19). Overall, the mechanism of basal α_4 integrin function on resting eosinophils has not been extensively studied.

An important molecule regulating integrin activation is the small GTPase Rap1 which has been extensively studied in the context of cellular activation (20). Small GTPases are intracellular

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Received for publication September 20, 2007. Accepted for publication February 15, 2008.

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[1] This work was supported by Netherlands Organisation for Scientific Research Grants NWO nr 916.036.051 (to L.H.U.) and UU2005-3659 (to M.B.).

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molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. This step is catalyzed by guanine nucleotide exchange factors (GEFs)[3] whereas the transition back into the GDP-bound state is catalyzed by GTPase-activating proteins. Studies using constitutively active and dominant-negative mutants of Rap1 in Jurkat cells have shown a role for this protein in both the function of α_4 and β_2 integrins binding to VCAM-1 and ICAM-1, respectively (20). Different physiological stimuli (chemokines, bacterial products) have been shown to be involved in Rap activation (4, 21) but basal Rap activity and its function in the context of cellular adhesion have not been addressed previously. In this study, we investigated the intracellular signaling pathways involved in the constitutive basal activity of α_4 integrins on eosinophils, using both pharmacological approaches in primary eosinophils and retroviral transduction strategies in $CD34^+$ hematopoietic progenitor-derived eosinophils.

Materials and Methods

Reagents

Immunomagnetic CD16, CD14, and CD3 beads and additional cell isolation tools were purchased from Miltenyi Biotec. Human serum albumin and pasteurized plasma solution was purchased from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Recombinant human TNF-α was purchased from Boehringer Mannheim. Isolation buffer contained PBS supplemented with pasteurized plasma solution (10% v/v) and sodium citrate (0.4% w/v). The phospholipase C (PLC) inhibitor U73122 (1-((6-(((17β-3-methoxestra-1,3,5(10)trien-17-yl)amino))hexyl))-1H-pyrrole-2,5-dione) and its inactive congener U73343 (1-((6-(((17β-3-methoxestra-1,3,5(10)trien-17-yl)amino))hexyl))-2,5-pyrrolidine-dione) were obtained from Calbiochem. The PI3K inhibitor LY294002, the MEK1/2 inhibitor PD98059, and the tyrosine kinase inhibitor genistein were obtained from Biomol Research Laboratories. The p38 MAPK inhibitor SB203580 was obtained from Alexis. Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) human serum albumin. All other materials were reagent grade.

Antibodies

The mAb HP2/1 (anti-α_4 integrin, CD49d) was purchased from Immunotech. mAb IB4 (anti-β_2 integrin) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection. Above-mentioned mAbs are functionally blocking Abs. Control Ab W6/32 (anti-HLA-A, -B, -C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection. In the perfusion experiments, mAbs were preincubated with eosinophil (4×10^6 cells/ml) at 10 μg/ml for 15 min. The cell suspensions were diluted twice with incubation buffer (final concentration of 5 μg/ml mAb at 2×10^6 cells/ml in incubation buffer), and the coverslips were placed directly in the flow system.

Isolation of eosinophils

Trisodium citrate (0.4% (w/v); pH 7.4) anticoagulated blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands) and Donor Service of the University Medical Center (Utrecht, The Netherlands). Mixed granulocytes were isolated as described previously (22). Mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of erythrocytes with an isotonic ice-cold NH_4Cl solution, granulocytes were washed and resuspended in isolation buffer. Eosinophils were purified from granulocytes by negative immunomagnetic selection using anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec) (23) and anti-CD3 and -CD14-conjugated microbeads were additionally added to avoid mononuclear cell contamination. Purity of eosinophils was >95%.

Isolation of $CD34^+$ cells

$CD34^+$ cells were isolated as previously described (24). Mononuclear cells were isolated from umbilical cord blood by density centrifugation over isotonic Ficoll solution (Pharmacia). MACS immunomagnetic cell separation (Miltenyi Biotec) using a bead-conjugated Ab against CD34, was used to isolate $CD34^+$ cells. $CD34^+$ cells were cultured in IMDM (Invitrogen Life Technologies) supplemented with 9% FCS, 50 μM 2-ME, 10 U/ml penicillin, 10 μg/ml streptomycin, and 2 mM glutamine at a density of 0.3×10^6 cells/ml. Cells were differentiated toward eosinophils by addition of stem cell factor (50 ng/ml), FMS-like tyrosine kinase 3 ligand (50 ng/ml), GM-CSF (0.1 nM/L), IL-3 (0.1 nM/L), and IL-5 (0.1 nM/L). Every 3 days, cells were counted and fresh medium was added to a density of 0.5×10^6 cells/ml. After 3 days of differentiation, only IL-3 and IL-5 were added to the cells.

Viral transduction of $CD34^+$ cells

A bicistronic retroviral DNA construct was used, expressing active RapV12 and an internal ribosomal entry site (IRES) followed by the gene encoding for enhanced GFP (EGFP) (LZRS-EGFP). LZRS-EGFP retrovirus was produced by transient transfection of the retroviral packaging cell line, Phoenix-ampho, by calcium phosphate coprecipitation. Cells were plated in 6-cm dishes, 24 h before transfection. A total of 10 μg of DNA was used per transfection. Medium was refreshed, 16 h after transfection. After an additional 24 h, viral supernatants were collected and filtered through a 0.2-μm filter. $CD34^+$ cells (day 1) were transduced in 24-well dishes precoated with 1.25 $\mu g/cm^2$ recombinant human fibronectin fragment CH-296 (RetroNectin; Takara) for 2 h, and 2% BSA for 30 min. Transduction was performed by addition of 0.5 ml of viral supernatant to 0.5 ml of medium containing 0.5×10^6 cells. Twenty-four hours after transduction, 0.7 ml of medium was removed from the cells and 0.5 mL fresh virus supernatant was added together with 0.5 ml of fresh medium. Cells were used in bead assays at day 3.

Depletion of intracellular-free Ca^{2+}

Intracellular-free Ca^{2+} was depleted as described (25). Eosinophils were suspended in Ca^{2+}-free incubation buffer supplemented with 1 mM EGTA. Indo-AM (Molecular Probes) was added to 1-ml aliquots of suspended cells (5×10^6/ml) at a final concentration of 1.5 μM. Cells were incubated for 40 min at 37°C. In some cases, thapsigargin (100 nM) was added during the last 10 min of the incubation to inhibit sarco/endoplasmic reticulum Ca^{2+} ATPases. thereby depleting intracellular Ca^{2+} stores (9, 26). Cells were washed once with incubation buffer containing EGTA.

Fluorescent-bead adhesion assay

The activation of α_4 integrins was determined by binding of VCAM-1-coated fluorescent microbeads to eosinophils. This fluorescent bead-based assay has been described by Geijtenbeek et al. (27). Briefly, TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes) were covalently coupled to streptavidin using 1-ethyl-3(−3-dimethylaminopropyl)-carbodiimide in MES buffer (pH 6). Then, beads were coated with biotin-SP-Affinipure goat anti-human Fc (γ) $F(ab')_2$ and subsequently coated with Fc-VCAM-1. Eosinophils were resuspended in incubation buffer, with or without Ca^{2+} and/or Mg^{2+} as indicated. Cells (40–50,000/well) were preincubated with control anti-HLA-ABC (W6/32) mAb, anti-α_4-integrin blocking mAb HP2/1 (10 μg/ml), or pharmacological inhibitors. The ligand-coated beads were washed twice and added together with the PMA, Mn^{2+}, or IL-5 (10^{-10} M) in a 96-well V-shaped-bottom plate. Next, the preincubated eosinophils were added and incubated for 30 min at 37°C. C5a (10^{-8} M) was added 15 min before the end of the experiment. The cells were washed and resuspended in incubation buffer (4°C) and kept on ice until measurement. In transduction experiments, cells were washed twice before the start of the experiment, and before analyses they were fixed with 2% paraformaldehyde. Binding of the fluorescent beads to the eosinophils was determined by flow cytometry using the FACSCalibur and results reported as the percentage of eosinophils positive for VCAM-1-coated beads.

Endothelial cells

HUVECs were isolated from human umbilical cord veins according to Jaffe et al. (28), with some minor modifications (29). The cells were cultured in endothelial cell growth medium-2 (BioWhittaker). Cell monolayers were grown to confluence in 5–7 days. Endothelial cells of the second or third passage were used in perfusion assays. HUVEC were activated by TNF-α (100 U/ml; 5–7 h, 37°C) before the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber as previously described by van Zanten et al. (29). This microchamber has a slit height of 0.2 mm and width

[3] Abbreviations used in this paper: GEF, guanine nucleotide exchange factor; PLC, phospholipase C; EGFP, enhanced GFP; DAG, diacylglycerol; CalDAG-GEF, calcium DAG-GEF; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm × 18 mm) with confluent HUVEC was mounted.

Eosinophil perfusion and evaluation

In vitro flow chamber experiments were performed as described (30). In short, eosinophils in suspension (2×10^6 cells/ml in incubation buffer) were aspirated from a reservoir through the perfusion chamber. Eosinophil perfusions were performed as individual runs under specific shear conditions at 37°C. Perfusion experiments were recorded on video tape and video images were evaluated for the percentage of rolling cells using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics Systems). The eosinophils, in the presence or absence of Abs (IB4, HP2/1 or W6/32; 10 μg/ml) or in the presence of the PLC inhibitor U73122 (10 μM) or the control analog U73343 (10 μM), were perfused during 3 min at shear stress 0.8 dyn/cm^2 to obtain an endothelial surface with firmly adhering and rolling eosinophils (13). Shear stress was increased to 2 dyn/cm^2 and recording of the images on video was started. For determining the percentage of rolling cells and rolling velocity, a sequence of 50 frames representing an adjustable time interval (Δt, with a minimal interval of 80 ms) was digitally captured. The cutoff value to distinguish between rolling and static adherent cells was set at 1 μm/s. With this method, static adherent, rolling and freely flowing cells (which were not in focus) could be clearly distinguished.

Rap1 activation assay

Rap1 activation was determined as described before (31). One milliliter of eosinophils ($2–5 \times 10^6$/ml) or CD34$^+$ progenitor cell-derived eosinophils ($2–5 \times 10^6$/ml) was incubated with U73343 or U73122 (2, 5, or 10 μM) for 15 min at 37°C as indicated. In some cases, cells were activated by C5a (10 s, 10^{-7} M). Then 0.5 ml of ice-cold 3× GST lysis buffer (final concentration of 1× GST lysis buffer: 200 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 2 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 1 mM benzamidine, 100 kIE/ml aprotinin, 1 μg/ml leupeptin, 1 mM vanadate, 1 mM DTT) was added and cell lysates were vortexed vigorously. Lysates were kept on ice for 10 min and clarified by centrifugation (10 min, 14,0000 rpm, 4°C). As a loading control for total Rap1, 1/30th (50 μl) of the cell lysate was reserved and supplemented with sample buffer and boiled (5 min). GST-RalGDSRBD (Ral guanine nucleotide dissociation stimulator Ras-binding domain)-containing bacterial lysate was precoupled to 50 μl of glutathione beads per sample and incubated for 30 min on a rotating wheel at 4°C. After coupling, GST-RalGDSRBD-coated beads were washed three times with GST lysis buffer, added to the cell lysate, and incubated for 1 h at 4°C. Samples were washed three times with GST lysis buffer and 20 μl of sample buffer was added and boiled (5 min). The samples (Rap1 pulldown and loading controls) were run on SDS-PAGE and transferred to polyvinylidene difluoride membranes (NEN). Rap1 was detected by a polyclonal rabbit Ab (sc-65; Santa Cruz Biotechnology) and HRP-coupled swine anti-rabbit using Enhanced Chemiluminescence Plus (Amersham Biosciences) and film (XOMAT; Kodak) or the Typhoon 9410 (Amersham Biosciences).

PCR of Rap GEFs

Isolated eosinophils ($2–4 \times 10^6$) and human leukemic Jurkat T cells (1×10^6), maintained as previously described (32) were washed three times with RNase-free PBS. Human brain tissue was obtained from The Netherlands Brain Bank (NBB; Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). This material was collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB.

Total RNA was extracted from cell pellets or tissue using a TRIzol kit (Invitrogen Life Technologies) as per the manufacturer's instructions. First-strand synthesis was performed with 1 μg of purified RNA, oligo(dT) primers and Superscript II (Invitrogen Life Technologies) at 42°C for 60 min. PCR was performed on cDNA for 33 cycles using specific forward and reverse primers previously described (all primers obtained from Invitrogen Life Technologies) (33): calcium diacylglycerol (DAG)-GEF I (CalDAG-GEF I) (annealing temperature 55°C): (forward (f)) TCCACTCCTCCAATACCG (reverse (r)) CAGGAC TATCACAGTTTCGT; CalDAG-GEF III (59°C): (f) CAAAGGCCTT GCGGTAATTG, (r) CCTTGGAAAGATCGATTGCT; PSD-95/DlgA/ZO-1 GEF 1 (PDZ-GEF 1) (54°C): (f) TGGAATGGAGTAGCGACT, (r) AGGCTGTTGAATATCGCG.

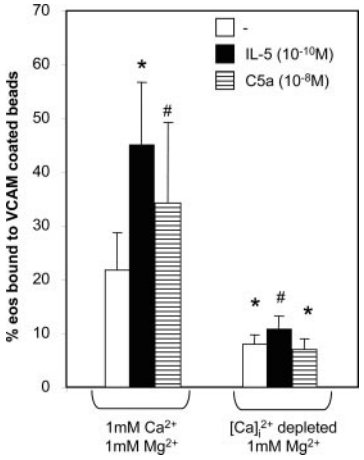

FIGURE 1. Role of intracellular Ca^{2+} on α_4-integrin function. Eosinophils were kept in a HEPES incubation buffer or were depleted from Ca^{2+} by incubation in HEPES/1 mM EGTA supplemented with 1.5 μM indo-AM for 40 min at 37°C (of which, the last 10 min was in the presence of 100 nM thapsigargin). Cells were washed and incubated with VCAM-1-coated beads for 30 min at 37°C in the absence of presence of IL-5 (10^{-10} M, 30 min) or C5a (10^{-8} M, 15 min) and analyzed by flow cytometry. Depicted is the percentage of eosinophils bound to VCAM-1-coated beads ± SEM. * and #, $p < 0.01$ and $p < 0.05$ compared with control bar, respectively, using an ANOVA test.

Statistical analysis

Results are expressed as mean ± SEM. Where indicated, statistical analysis was performed using ANOVA with patient groups as main factors. A post-hoc analysis was done via the least significant difference test under the protection of a significant group effect.

Results

Depletion of intracellular Ca^{2+} decreases α_4 integrin function

We investigated the role of intracellular Ca^{2+} homeostasis in the regulation of basal α_4 integrin function by studying the binding of eosinophils to VCAM-1-coated fluorescent beads (Fig. 1). Eosinophils were depleted of intracellular calcium using EGTA, indo-AM, and thapsigargin. Extracellular cation requirements for integrin function were maintained by the presence of 1 mM Mg^{2+} in the incubation buffer as extracellular Mg^{2+} has been shown to support α_4-integrin activity on several cell lines in the absence of extracellular Ca^{2+} (34). We have similar data for eosinophils (data not shown). Despite the presence of extracellular Mg^{2+}, depletion of intracellular Ca^{2+} decreased eosinophil α_4 integrin binding by ~75%. The basal level of eosinophil α_4-integrin activity in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} could be further augmented by stimulation with cytokines (IL-5, 10^{-10} M) and chemoattractants (C5a, 10^{-8} M). However, neither C5a nor IL-5 were able to up-regulate α_4-integrin function following intracellular Ca^{2+} depletion, demonstrating that both basal and induced α_4-integrin binding are modulated by Ca^{2+} homeostasis. Activation of Ca^{2+}-depleted eosinophils by 0.5 mM Mn^{2+} was not able to induce binding to VCAM-coated beads (data not shown). Furthermore, the binding of IL-5-stimulated eosinophils to VCAM was totally abrogated by preincubation with the blocking Ab HP2/1 (data not shown) suggesting that alternative IL-5-induced binding to VCAM which has been shown to be mediated by β_2 integrins (35) does not play a role in our system. Also, because we used the anti-α_4-integrin blocking Ab HP2/1, we cannot discriminate between $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins.

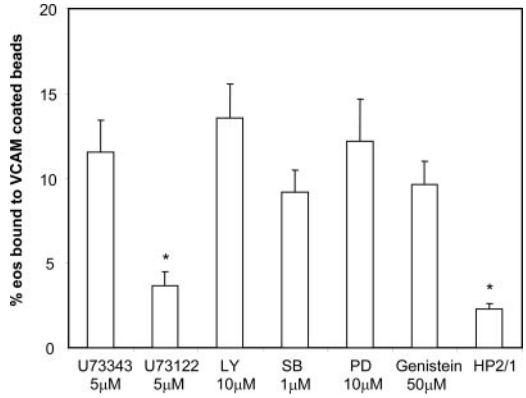

FIGURE 2. Effect of pharmacological inhibitors of intracellular signaling pathways on α_4-integrin function. Eosinophils, preincubated with pharmacological inhibitors (PLC inhibitor; U73122, PI3K inhibitor; LY294002, p38 MAPK inhibitor; SB203580, MEK inhibitor; PD-98059), control compounds (U73343), or blocking anti-α_4-integrin Abs (HP2/1) for 15 min, 37°C were added to VCAM-1-coated beads for 30 min at 37°C and analyzed by flow cytometry. Depicted is the percentage of eosinophils bound to VCAM-1-coated beads ±SEM. *, $p < 0.001$ compared with the first bar using an ANOVA test.

Inhibition of PLC decreases basal α_4-integrin function

PLC enzymes are important regulators of intracellular Ca^{2+} homeostasis. Therefore, we studied the role of these and other signaling proteins in controlling basal α_4 function of human eosinophils (Fig. 2). Preincubation of eosinophils with the PLC inhibitor U73122, in contrast to its control compound U73343, significantly inhibited the basal binding of α_4 integrin on resting eosinophils to levels observed in the presence of blocking anti-α_4 Abs. A dose-response curve of U73122 revealed that 5 and 10 μM both inhibited α_4 integrin function whereas 2 μM was partly effective and 1 μM was not effective (data not shown). Involvement of PLC in maintaining basal α_4-integrin activation was selective, as α_4-integrin function was not significantly influenced by inhibitors of PI3K (LY294002, 10 μM), p38 MAPK (SB302580, 1 μM (and 10 μM, data not shown)), MEK (PD98059, 10 μM), or tyrosine kinases (genistein, 50 μM). The effects of PLC inhibition on α_4-integrin function were not due to modulation of integrin expression, as FACS analysis revealed that U73122 did not influence α_4-integrin surface expression (data not shown). These results suggest that inhibition of PLC plays an important and selective role in activation of α_4 integrins on resting eosinophils.

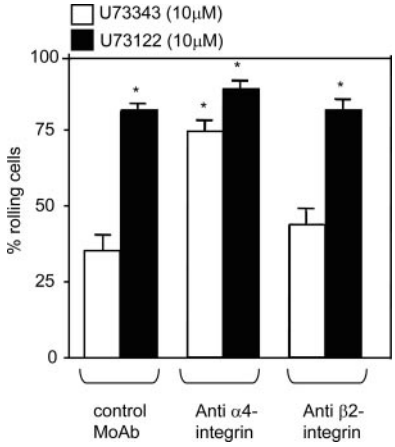

FIGURE 3. Effect of phospholipase C inhibitor on rolling interactions of eosinophils with TNF-α-activated HUVECs. Eosinophils were preincubated with control (W6/32) or blocking Abs against α_4 integrins (HP2/1) or β_2 integrins (IB4) and control compound U73343 (10 μM) or PLC inhibitor U73122 (10 μM) as indicated. Cells were perfused on 5–7 h TNF-α-activated HUVECs at 0.8 dyn/cm^2 for 3 min; subsequently, shear was increased to 2 dyn/cm^2 and images were recorded. Off line, the percentage of rolling cells was calculated and depicted ± SEM. *, $p < 0.001$ compared with control bar as determined by ANOVA test.

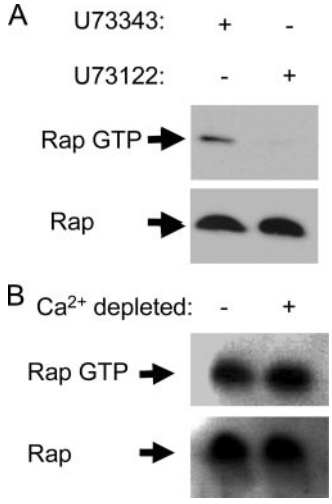

FIGURE 4. Basal active Rap1 levels in eosinophils are sensitive to PLC inhibition but not for Ca^{2+} depletion. Eosinophils were preincubated either with (*A*) the PLC inhibitor U73122 (10 μM) or its control peptide U73343 (10 μM) during 15 min or (*B*) depleted from Ca^{2+} by incubation in HEPES/1 mM EGTA supplemented with 1.5 μM indo-AM for 40 min at 37°C (of which, the last 10 min was in the presence of 100 nM thapsigargin followed by a pulldown of Rap1-GTP). A representative experiment of three is shown.

Inhibition of PLC decreases α_4 integrin-mediated basal adhesion to activated endothelial cells under flow conditions

We next investigated whether PLC regulated α_4 integrin-mediated attachment of eosinophils to activated endothelial cells under physiological conditions, using an in vitro flow chamber model. Both E-selectin and VCAM-1 play important roles in the initial attachment of eosinophils to TNF-α-activated HUVECs (13). In this model system, E-selectin predominantly mediates rolling interactions, while VCAM/α_4-integrin interactions are responsible for direct capture and firm arrest of eosinophils. As a result, inhibition of α_4 integrins enhances the fraction of eosinophils rolling on E-selectin of TNF-α-activated HUVEC. In control experiments,

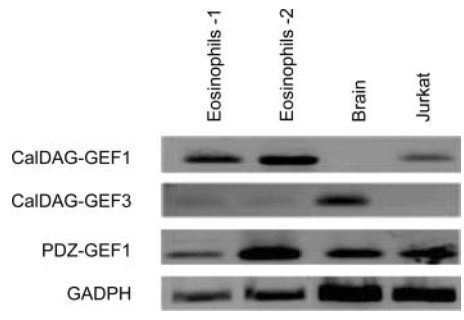

FIGURE 5. Expression of Rap GEFs in resting eosinophils. PCR analysis of GEF and control GAPDH expression in freshly isolated, resting eosinophils from two independent donors was performed using transcript-specific primers. Human brain and Jurkat T cell RNA was used as positive controls for GEF expression.

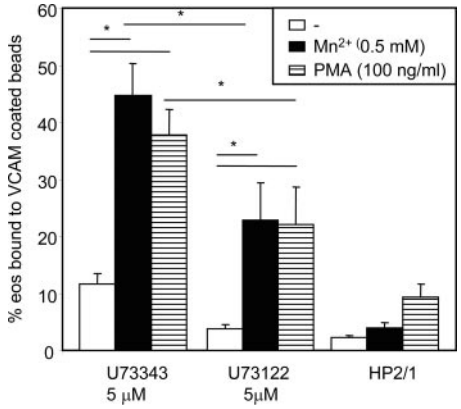

FIGURE 6. Mn^{2+} and PMA overcome the U73122-induced inhibition of eosinophil function. Eosinophils—preincubated with PLC inhibitor U73122 or the control compounds U73343 or blocking anti-α_4-integrin Abs (HP2/1) for 15 min, 37°C—were added to VCAM-1-coated beads for 30 min at 37°C in the presence or absence of 1 mM Mn^{2+} or 100 ng/ml PMA. The percentage of eosinophils bound to VCAM-1-coated beads ± SEM is depicted. *, $p < 0.001$ compared with the unstimulated sample using an ANOVA test.

inhibition of α_4 integrins on resting eosinophils with mAb HP2/1 enhanced the percentage of rolling from 35 ± 6% (control mAb) to 74 ± 4% (Fig. 3). In contrast inhibition of β_2 integrins by the mAb IB4 did not significantly change the percentage of rolling cells. This suggests that α_4, but not β_2, integrins are functionally active on resting, freshly isolated eosinophils. Inhibition of PLC dramatically increased the percentage of rolling cells from 35 ± 6% (control compound U73343) to 81 ± 3% (PLC inhibitor U73122). Thus, inhibition of either PLC or α_4 integrins resulted in a similar increase in the percentage of rolling cells. These results strongly support the hypothesis that inhibition of PLC by U73122 deactivates α_4 integrins on resting eosinophils.

Activation of Rap1 in resting eosinophils correlates with basal α_4-integrin activity

Because the GTPase Rap1 has been shown to play a role in several cases of agonist-induced integrin activation (4, 20, 21, 36), we tested the hypothesis that Rap1 may also regulate basal α_4-integrin function in resting eosinophils. Active GTP-bound Rap1 was readily precipitated from lysates of resting eosinophils (Fig. 4*A*). Rap1 activation was further enhanced by stimulation of eosinophils with C5a (10^{-8} M, 10 s, data not shown) as previously described (4). Pretreatment of resting eosinophils with PLC inhibitor completely abolished basal activation of Rap1 (Fig. 4*A*). However, basal Rap1 activation was maintained independently of intracellular Ca^{2+} because depletion of Ca^{2+} had no effect on Rap1 GTP-binding (Fig. 4*B*). Previously, we have reported that the C5a-induced activation of Rap-GTP in eosinophils is inhibited when the cells were incubated with the PLC inhibitor U73122 (4). This, along with our present study, indicates a critical role for PLC in the regulation of both basal and agonist-induced Rap1 activity in eosinophils. Two activators of Rap1, the GEFs CalDAG-GEF 1 and 3, are known to be regulated by PLC-dependent generation of DAG and intracellular calcium. Analysis of eosinophil mRNA indicated that CalDAG-GEF 1 was strongly expressed in resting eosinophils, and low but detectable levels of CalDAG-GEF 3 were also observed (Fig. 5). These GEFs may provide a mechanistic link between PLC and Rap activation in resting eosinophils.

U73122-induced abrogation of α_4-integrin function is rescued by Mn^{2+} and PMA treatment

We next investigated whether protein kinase C (PKC), a downstream effector of the PLC, might overcome U73122-induced inhibition of integrin activation. PMA was used to bypass cellular receptors and directly activate the intracellular PKC pathway whereas the divalent cation Mn^{2+} was used to activate integrins from the outside. Fig. 6 shows that addition of Mn^{2+} or PMA resulted in increased binding of eosinophils to VCAM-1-coated beads in control, U73343-treated cells. Both Mn^{2+} and PMA stimulation rescued the U73122-induced inhibition of binding to VCAM-coated beads. However, Mn^{2+} and PMA-induced α_4-integrin activation in U73122-treated cells did not reach similar levels as U73343-treated cells ($p < 0.005$).

Homeostatic Ca^{2+} levels regulate Rap1-dependent α_4-integrin function in eosinophils

As our experiments indicated that homeostatic intracellular Ca^{2+} levels were essential for basal activation of α_4 integrins, but dispensable for Rap1 activation, we investigated whether Ca^{2+} may operate downstream of Rap1. Because retroviral transduction of terminally differentiated eosinophils was technically not successful, 3-day-old $CD34^+$ cells were used to test the importance of active Rap1 in the control of basal α_4 integrin activation. In initial

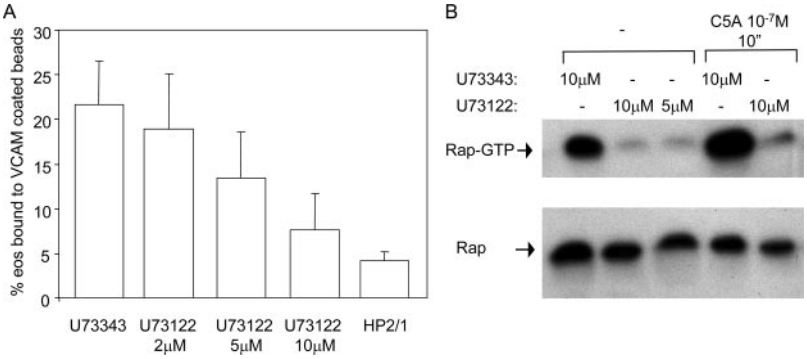

FIGURE 7. α_4 integrin function and basal Rap activation on hematopoietic progenitor-derived ex vivo differentiated eosinophils (day 17) are downregulated by PLC inhibition. $CD34^+$ progenitor stem cells were cultured for 17 days. Ex vivo-differentiated eosinophils—preincubated with phospholipase C inhibitor U73122 or the control compounds U73343 or blocking anti-α_4-integrin Abs (HP2/1) for 15 min, 37°C—were added to VCAM-1-coated beads for 30 min at 37°C and analyzed by flow cytometry. *A*, The percentage of ex vivo-differentiated eosinophils bound to VCAM-1-coated beads is depicted ($n = 3$–4, ±SEM). *B*, Ex vivo-differentiated eosinophils were preincubated with phospholipase C inhibitor U73122 or the control compounds U73343 for 15 min, 37°C and additionally stimulated or not with C5a (10^{-7} M) for 10 s. Subsequently, a rap pulldown was performed. Representative experiment of three is shown.

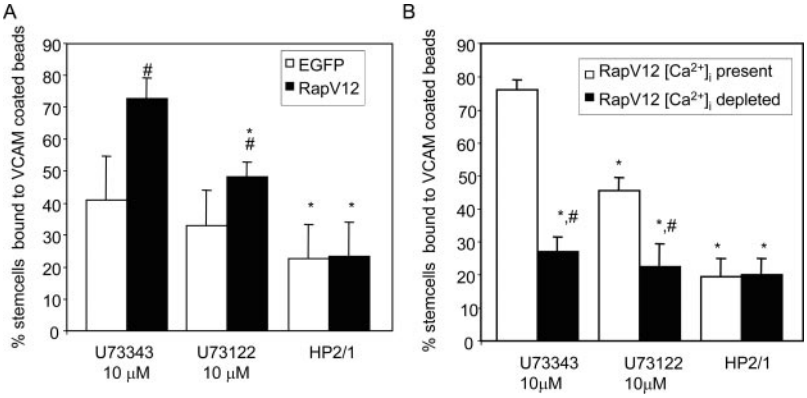

FIGURE 8. Ca^{2+} depletion inhibits binding to VCAM-1 beads in the presence of constitutively active Rap. $CD34^+$ hematopoietic progenitors were transduced with EGFP-LZRS or EGFP-RapV12-LZRS virus at days 1 and 2 and measured at day 3. *A*, Cells were preincubated with PLC inhibitor U73122, the control compounds U73343, or blocking anti-α_4-integrin Abs (HP2/1) for 15 min, 37°C and added to VCAM-1-coated beads for 30 min at 37°C and analyzed on EGFP-positive cells by flow cytometry. The percentage of $CD34^+$ cells bound to VCAM-1-coated beads ± SEM ($n = 3$) is depicted. *, $p <$ 0.005 compared with the corresponding U73343-treated group; #, $p < 0.01$ of Rap V12 cells compared with EGFP cells with similar treatment using an ANOVA test. *B*, EGFP-RapV12-transduced cells were depleted from intracellular Ca^{2+} or not and subsequently a VCAM-1 bead assay was performed. The percentage of $CD34^+$ cells bound to VCAM-1-coated beads ± SEM ($n = 4$–5) is depicted. *, $p < 0.005$ compared with U73343-treated EGFP-RapV12-transduced cells in the presence of $[Ca^{2+}]_i$. #, $p < 0.005$ of the $[Ca^{2+}]_i$-depleted group compared with the same treatment in presence of $[Ca^{2+}]_i$ using an ANOVA test.

experiments, we confirmed that α_4 integrin on ex vivo-differentiated eosinophils was regulated by the same mechanisms as in mature blood eosinophils (Fig. 7). Basal activation of α_4 integrins is readily observed in ex vivo-differentiated eosinophils. This basal activity was abrogated upon treatment with the PLC inhibitor U73122 but not the control compound U73343 (Fig. 7*A*), suggesting that α_4-integrin function is regulated by the same mechanism in ex vivo-differentiated eosinophils and mature eosinophils. Furthermore, integrin activation was further increased by divalent cation Mn^{2+} (data not shown) confirming the intermediate affinity of α_4 integrins on resting, cultured ex vivo-differentiated eosinophils. Additionally, basal Rap GTP activity in ex vivo-differentiated eosinophils was inhibited upon treatment with the PLC inhibitor (Fig. 7*B*). C5a stimulation further increased Rap activity which was down-regulated by pretreatment of the PLC inhibitor U73122 compared with the control compound U73343. When $CD34^+$ hematopoietic progenitors were retrovirally transduced to ectopically express constitutively active Rap1 (RapV12), Rap1 signaling resulted in an increase in α_4-integrin function compared with control EGFP-transduced cells (Fig. 8*A*). Furthermore, inhibition of PLC was able to down-regulate α_4-integrin function in the RapV12-transduced cells (Fig. 8*A*). In line with our hypothesis, depletion of intracellular Ca^{2+} in $CD34^+$ cells ectopically expressing RapV12 reduced the percentage of cells binding to VCAM beads (Fig. 8*B*). $CD34^+$ EGFP-transduced control cells which were depleted from intracellular Ca^{2+} showed similar low binding to VCAM-coated beads as the RapV12-expressing cells (data not shown). These results suggest that both activation of Rap1 and homeostatic cytosolic-free Ca^{2+} are essential for basal integrin function in resting eosinophils.

Discussion

In this study, we have demonstrated that basal activity of α_4 integrins on primary eosinophils is dependent upon normal homeostatic concentrations of intracellular-free Ca^{2+} (±100 nM) (25) and activity of the small GTPase Rap1. Basal α_4-integrin activity correlated with Rap1 activation in the sense that inhibition of PLC resulted in both loss of α_4-integrin activity and Rap GTP loading. Furthermore, depletion of intracellular Ca^{2+} had no effect on Rap1 activation status, but ablated α_4-integrin function. In corroboration of this, intracellular Ca^{2+} depletion abrogated RapV12-induced increase in α_4-integrin activity. Taken together, our data provide evidence that resting eosinophils actively use a Ca^{2+}-dependent system to maintain a functional pool of α_4 integrins.

The existence of a basal activation state of α_4 integrin on several leukocyte types has been extensively reported (10–13). However, this is the first report addressing a role for the PLC/intracellular Ca^{2+} pathway for the maintenance of basal α_4-integrin activation on primary eosinophils. In contrast to T cells and basophils, which were shown to depend on *src* kinases for their basal activation state of α_4 integrins (16, 17), eosinophil α_4-integrin activity was not inhibited by either PP1 (which inhibits tyrosine kinases of the *src* kinase family) nor the general tyrosine kinase inhibitor genistein (data not shown and Fig. 3). This is consistent with a study showing that genistein treatment of eosinophils did not affect α_4 integrin-mediated adhesion (19). In the latter study, tyrosine kinase signaling was shown to be important in integrin outside-in signaling-induced eosinophil effector functions. In another study (18), an epidermal growth factor receptor kinase inhibitor tyrphostin B46 was shown to inhibit α_4-integrin function on resting eosinophils leaving the possibility of the involvement of a nongenistein-sensitive kinase pathway. Another intracellular pathway which has been suggested to play a role in spontaneous eosinophil adhesion to fibronectin is the ERK1/2-cPLA2 pathway (37). We have not tested cPLA2 inhibitors, however, we did not find any inhibition of α_4-integrin function by the MEK1/2 inhibitor PD98059 suggesting that ERK1/2 and downstream effectors are not involved in basal α_4-integrin activity. Furthermore, we found that pharmacological inhibition of the PI3K and p38 MAPK pathways also did not affect basal α_4-integrin activity. Similarly, Feigelson et al. (16) showed that basal α_4-integrin activity on basophils was not sensitive for PI3K inhibitors, as determined in aggregation assays. In our studies, only inhibition of PLC abrogated binding of eosinophil α_4 integrin to VCAM-coated beads. Thus, eosinophils seem to regulate basal α_4-integrin activity via a PLC-dependent pathway which is different from other leukocyte types.

Because PLC activity leads to increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and DAG and PLC inhibition blocked α_4-integrin function, we addressed the role of metabolic products of PLC in mediating adhesion of resting eosinophils. Depletion of

intracellular Ca^{2+} decreased α_4-integrin function but did not lower Rap1-GTP levels compared with control cells. In contrast, inhibition of PLC decreased both of these experimental parameters. Furthermore, RapV12-mediated α_4-integrin activation in $CD34^+$ hematopoietic progenitor cells was totally abrogated upon depletion of intracellular Ca^{2+}. These data are consistent with different, not mutually exclusive, hypotheses: 1) Rap activity is essential but not sufficient for α_4-integrin function and 2) in contrast to the Ca^{2+} pathway the DAG pathway downstream of phospholipase C is involved in Rap activation and 3) intact homeostasis of intracellular Ca^{2+} is important for basal integrin activity. Our findings showing the lack of down-regulation of basal Rap levels upon Ca^{2+} depletion is consistent with findings in Jurkat T cells in which BAPTA-treated cells fail to block TCR or stromal cell-derived factor-1-induced Rap activation, whereas the PLC inhibitor U73122 does so effectively (38). Also, in neutrophils it has been shown that intracellular Ca^{2+} depletion does not abrogate chemokine-induced Rap activity (31). We found that both CalDAG-GEF I and CalDAG-GEF III, GEFs for Rap1 which contain Ca^{2+}- and DAG-binding domains are expressed in eosinophils. It has been suggested that CalDAG-GEF I and III differ in their dependency on Ca^{2+} and DAG. CalDAG-GEF I is thought to be more Ca^{2+} dependent while activation of CalDAG-GEF III relies more heavily upon DAG (reviewed in Ref. 39). Because we observed no decrease in basal Rap activity upon Ca^{2+} depletion it might be that in eosinophils DAG levels but not Ca^{2+} are necessary for basal Rap1 activity, suggesting a role for CalDAG-GEF III in this process. Indeed, PMA was able to induce α_4-integrin activation in the presence of the PLC inhibitor. A physiological role for CalDAG-GEFs in integrin activation has been illustrated previously by the observation that CalDAG-GEF $I^{-/-}$ mice have severe bleeding disorders due to defects in inside-out signaling of $\alpha_{IIb}\beta_3$ on platelets (40).

In U73122-treated eosinophils, PMA and Mn^{2+} did not result in a total rescue of VCAM binding when compared with U73343-treated cells. Why this is we do not know, but we can speculate on the underlying mechanism. PKC is known to be partially dependent on Ca^{2+} for optimal function. Therefore, impaired Ca^{2+} homeostasis induced by the inhibition of PLC by U73122 may prevent optimal PMA activity in eosinophils. Supporting this hypothesis is the finding that Rap cannot be activated fully in U73122-treated eosinophils stimulated with PMA (data not shown) which also parallels the observation with binding of VCAM beads. The effect of U73122 on Mn^{2+}-induced activation can be explained along similar lines. It is known that dominant-negative Rap down-regulates Mn^{2+}-induced integrin activation (32). Apparently, inhibition of the Rap pathway functionally limits the pool of integrin which can be induced to a high-affinity conformation by Mn^{2+}. Because we know that U73122 is inhibiting Rap, it is conceivable that Mn^{2+}-induced integrin activation is partly sensitive for any signal acting through Rap.

Because the U73122 compound inhibits all isoforms (β, γ, δ, and ε) of PLC, we are unable to conclude from our studies which isoform is involved in the basal activity of α_4 integrins. However, because the PLCγ isoforms are dependent on tyrosine phosphorylation, and tyrosine kinase inhibitors did not have any effect on eosinophil α_4-integrin function, it is unlikely that these are involved in resting, nonactivated eosinophils. In contrast to a cell in resting condition, several studies have shown that after activation by cytokines or T or B cell Ag receptor stimulation, PLCγ activation results in Rap1 and integrin activation (38, 41) (42). As an active mutant of Rap (Rap1-63E) has been reported to overcome U73122-induced inhibition of β_1-integrin activity (41), Rap1 has been placed downstream of PLC but upstream of the integrin. In line with these data, we show in Fig. 4 that U73122 treatment resulted in inhibition of Rap, thereby placing Rap activation downstream of PLC which fits the general view of the existence of a PLC–Ca^{2+}/DAG–CalDAG-GEF–Rap-integrin axis (reviewed in Ref. 43). However, in our RapV12-transduced primary stem cells, inhibition of PLC resulted in a partial inhibition of binding to VCAM, placing PLC also downstream of Rap. Interestingly, PLCε is the only PLC isoform which acts as a downstream effector of Rap-GTPases. This may support activation of Ca^{2+}- and DAG-induced pathways downstream of Rap, leading to integrin activation (44, 45). However, a study by Lad et al. (46) showed that H-*ras*-induced PLCε activation resulted in a down-regulation of integrin activity which would be in contrast to this hypothesis.

Taken together, this is the first study that demonstrates that basal α_4-integrin function on eosinophils is PLC and $[Ca^{2+}]_i$ dependent and that Rap-induced α_4-integrin activity requires intact intracellular Ca^{2+} homeostasis. These observations may contribute to a better understanding of α_4 integrins under homeostatic conditions. α_4 integrins are highly attractive drug targets in several inflammatory diseases. The greatest success has been the use of the anti-α_4-integrin monoclonal natalizumab for treatment of multiple sclerosis and inflammatory bowel disease. However, rare complications of progressive multifocal leukoencephalopathy have been described with this compound (47). To improve α_4 integrin-based therapies, and to prevent deleterious side effects, more insight into the mechanisms of α_4 integrins on different cell types is required.

Acknowledgments

We are grateful to Leo Houben for isolating eosinophils and to Pieter Zanen for help with statistical analysis.

Disclosures

The authors have no financial conflict of interest.

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