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β_2-Integrin-Mediated Adhesion and Intracellular Ca^{2+} Release in Human Eosinophils

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Abstract Human eosinophils spontaneously adhere to various substrates in the absence of exogenously added activators. In the present study a method was developed for characterizing eosinophil adhesion by measuring changes in impedance. Impedance measurements were performed in HCO_3-buffered HybriCare medium maintained in a humidified 5% CO_2 incubator at 37°C. Impedance increased by more than 1 kΩ within minutes after eosinophils made contact with the substrate, reaching a peak within 20 min. Blocking mobilization of intracellular [Ca^{2+}] that precedes adhesion with BAPTA-AM (10 μM) completely inhibited the rise in impedance as well as the changes in cell shape typically observed in adherent cells. However, lowering the extracellular [Ca^{2+}] with 2.5 mM EGTA did not inhibit the increase in impedance. Pretreatment with anti-CD18 antibody to block substrate interactions with β_2-integrins, or jasplakinolide (2 μM) to block actin reorganization, abolished the increase in impedance and adherent morphology of the cells. Exposure of eosinophils to the phosphatidylinositol 3 kinase inhibitor LY294002 (5 μM) or treatment with protein kinase C zeta pseudosubstrate to competitively inhibit activity of the enzyme significantly reduced the increase in impedance and inhibited the cell spreading associated with adhesion. These results demonstrate a novel method for measuring eosinophil adhesion and showed that, following formation of a tethered attachment, a rapid increase in intracellular [Ca^{2+}] precedes the cytoskeletal rearrangements required for cell shape changes and plasma membrane-substrate interactions associated with adhesion.

Keywords PI-3 kinase · β_2-Integrin · Actin · Degranulation · Major basic protein

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Eosinophil recruitment is often associated with allergic diseases such as atopic dermatitis and asthma as well as with helminthic infections (Gleich 1990). Many types of signaling molecules activate eosinophils, including specific cytokines (IL-5, IL-3, and GM-CSF), secretory IgA (sIgA), IgG-coated objects, lipoxygenase products such as leukotriene B_4, and lipid mediators including platelet activating factor (Abu-Ghazaleh et al. 1992; Bankers-Fulbright et al. 2004; Rothenberg and Hogan 2006). Each stimulus has a distinct signaling pattern, and whether or not these stimuli use common pathways to activate eosinophils remains an active area of investigation. In particular, IL-5 is thought to play a critical role in most eosinophil-associated diseases (Jenei et al. 2006; Kato et al. 2005b; Sano et al. 2005; Someya et al. 1997; Yamaguchi et al. 2006). For example, animal and human studies using anti-IL-5 antibody treatment show inhibition of eosinophilia and bronchial hyperreactivity (Walsh 2001). Eosinophils initially respond to activation by

producing superoxide anion in much greater amounts than observed in neutrophils or macrophages (Rothenberg and Hogan 2006; Someya et al. 1997). Activation of plasma membrane NADPH oxidase is responsible for the extracellular generation of superoxide anions (Bankers-Fulbright et al. 2001; Someya et al. 1997). Following the superoxide burst, the granule components of the eosinophil are usually released over a time period requiring several hours. Eosinophil granules contain toxic proteins that are thought to play an important role in the pathogenesis of asthma and other allergic diseases involving eosinophilic inflammation (Denzler et al. 2001; Walsh 2001).

An important step that precedes eosinophil activation is adhesion (Horie and Kita 1994; Kato et al. 1998). Previous studies have demonstrated that adhesion involving β_2-integrin engagement (CD11b/CD18) results in tyrosine phosphorylation of Cbl, a 120-kDa proto-oncogene (c-*cbl*) product known to play an important role in signal transduction for several receptors expressed by immune cells including polypeptide growth factor receptors and antigen receptors present on T cells and B cells (Donovan et al. 1994; Kim et al. 1995; Odai et al. 1995). Tyrosine phosphorylation of Cbl enhances binding to SH2 or SH3 domain-containing proteins such as phosphatidylinositol 3 (PI-3) kinase and PLC-γ (Odai et al. 1995). In eosinophils, β_2-integrin ligation and tyrosine phosphorylation of Cbl have been associated with an increase in inositol phosphate (IP) production that was blocked by genistein, a broad-spectrum protein tyrosine kinase inhibitor (Kato et al. 1998). These results indicated that β_2-integrin-dependent phosphorylation of Cbl was involved in activation of PLC-γ, leading to the production of IP_3 and diacylglycerol. Release of IP_3 would be expected to produce an increase in $[Ca^{2+}]_i$, which would likely play a role in the initial stages of adhesion.

In the present study the effects of β_2-integrin engagement on intracellular calcium mobilization and eosinophil adhesion in the absence of exogenously applied signaling molecules were investigated. Spreading of individual cells was tracked by measuring changes in surface area contact with the substrate. Simultaneous measurements of intracellular $[Ca^{2+}]_i$ were then correlated with eosinophil morphology changes to reveal the temporal relationship between β_2-integrin ligation, Ca^{2+} mobilization, and adhesion. To study adhesion in populations of eosinophils, a method was developed to monitor changes in impedance between the plasma membrane and the substrate as an approach for measuring increases in surface area contact and the relative intensity of cell membrane interactions with the substrate. Using this approach the contributions of PI-3 kinase and protein kinase C zeta (PKCζ) were determined, along with the effects of inhibition of extracellular Ca^{2+} influx and intracellular Ca^{2+} mobilization on the process of adhesion.

Materials and Methods

Materials

Unless otherwise noted in the text, all chemicals and reagents were obtained from Sigma (St Louis, MO). Fura-PE3/AM was purchased from Calbiochem (La Jolla, CA). Alexa Fluor 488 and Hank's balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA) and Hybri Care medium was purchased from ATCC (Manassas, VA). Normal goat IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD18 antibody was purchased from BD Biosciences Pharmingen (San Diego, CA) and anti-CD16 magnetic beads were purchased from Miltenyi Biotech (Auburn, CA). Alpha calf serum was purchased from HyClone Laboratories (Logan, UT). PKCζ myristoylated pseudosubstrate was purchased from Calbiochem (San Diego, CA).

Eosinophil Isolation

Human eosinophils were isolated from venous human blood as described previously (Hansel et al. 1991; Ide et al. 1994). Heparinized blood was collected from atopic and nonatopic volunteers and an equal volume of PIPES buffered solution was added (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4). The diluted blood was layered onto a Percoll gradient (density, 1.085 g/ml). After centrifugation (560 *g*, 30 min at 4°C), the plasma and Percoll layers were removed by aspiration. Tubes were wiped to remove contaminating leukocytes and red cells lysed by osmotic shock with distilled water. The remaining pellet, containing neutrophils and eosinophils, was incubated for 30 min with an equal volume of anti-CD16 magnetic beads on ice. After incubation, the cell mixture was diluted with PIPES buffered solution and 1% calf serum and eluted through a steel-wool column suspended in a strong magnet. Column eluate (14 ml) was collected and the number and purity of eosinophils were determined by staining with Randolph's stain. Eosinophil purity was always >95% and the major contaminating cells were neutrophils.

Determining Adhesion by Single-Cell Imaging

Measurements of eosinophil spreading were performed in serum-free Hybri Care medium containing 0.05% bovine serum albumin in the absence (control) or presence of anti-CD18 blocking antibody (10 μg/ml). Images of cells were obtained using a Nikon Diaphot inverted microscope (400×) with Hoffmann modulation optics at 25°C. Serial digital images were acquired at 20-s intervals and subsequently analyzed using Image J software (NIH, Bethesda

MD). Area analysis involved outlining the plasma membrane of the cell in each frame and referencing the measured pixel area to the initial area of the cell at time 0.

Tracking Adhesion by Measuring Impedance

Substrate-induced adhesion was measured using Electric Cell-substrate Impedance Sensing (ECIS; Applied Bio-Physics, Troy, NY), which continuously monitors changes in impedance following attachment to the substrate. Specially designed chambers were used that contain a 250-μm gold film electrode that passes a brief alternating current (~1 μA) which allows for measurement of impedance as eosinophils undergo morphologic changes associated with adhesion. This approach provided an automated measurement system that was correlated with changes in cell morphology recorded as digital images using an inverted Nikon Diaphote fluorescence microscope. Typical duration of an experiment was 1 h. Cells were suspended in serum-free Hybri Care medium (5×10^6 cells/ml, with 0.2 ml cell suspension added to each well) containing 0.05% bovine serum albumin in the presence or absence (control) of various inhibitors as indicated in the figure legends.

Intracellular [Ca^{2+}] Measurements

Eosinophils were plated onto chamber slides that allowed for perfusion and ratio imaging. Eosinophils were washed with HBSS containing 10 mM glucose, 10 mM HEPES, pH 7.4. The cells were then loaded with 10 μM fura-2-AM (Invitrogen, Eugene, OR) for 45 min at 37°C, washed in HBSS, and transferred to the stage of a Nikon Diaphot inverted microscope with an epifluorescence attachment. The chamber slide was perfused with HBSS (2 ml/min) at 25°C. Fluorescence changes in single cells were visualized using a Nikon UV-fluor 40× oil-immersion objective. Image acquisition was controlled using Image-1 Metamorph software (Universal Imaging, Westchester, PA). $[Ca^{2+}]_i$ was measured as the ratio of fluorescence emitted at 510 nm when the cells are alternately excited at 340 nm and 380 nm [F_{340}/F_{380}]. $[Ca^{2+}]_i$ was calculated according to the calibration equations provided by Grynkiewicz et al. (1985).

Immunocytochemistry for MBP

Immediately after measuring impedance, the chamber slides containing eosinophils were placed on ice, the medium was removed, and ice-cold 1% paraformaldehyde was added for 30 min to fix the cells. After 30 min, chambers were washed 3× with ice-cold PBS, 10% normal goat IgG was added to each chamber, and chambers were stored overnight at 4°C. The next day chambers were washed 3 × 15 min with 200 μl 1× PBS per chamber, followed by incubation with anti-MBP (kindly provided by Dr. Gerald Gleich, University of Utah) or normal rabbit IgG serum (40 μg/ml) for 30 min at 37°C. Chambers were washed 3 × 15 min with PBS as above and 200 μl chromotrope 2R was added to each chamber for 30 min at room temperature. Chambers were washed 4 × 15 min with 200 ml PBS as above and stained with 200 μl FITC goat anti-rabbit IgG (40 μg/ml) for 30 min at 37°C. Chambers were washed 3 × 15 min with 1× PBS and the plastic chambers were removed from the glass slide. Specimens were mounted with 10% 1× PBS/90% glycerol mounting medium containing 0.1% *p*-phenylenediamine; coverslips were then applied prior to microscopy.

Actin Labeling and Confocal Imaging

Purified human eosinophils were resuspended to 0.5×10^6 cells/ml in HBSS (with 10 mM HEPES and 0.1% BSA, pH 7.4) and 200 μl of cell suspension was added to each well of a Lab-Tek eight-well chambered coverglass slide (Nalge Nunc International Corp., Naperville, IL). Eosinophils were treated with jasplakinolide, LY294,002, PKCζ pseudosubstrate, cytochalasin D, anti-CD18, or medium control for 30 min at 37°C (5% CO_2). Treated cells were fixed, permeabilized, and stained with Alexa Fluor 488 phalloidin according to product instructions. Following staining, cells were washed and covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to prevent Alexa Fluor 488 bleaching and DAPI (4′,6-diamidino-2-phenylindole) for nuclear staining. Stained cells were examined using a 63× water-immersion objective on a Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

Statistics

Comparisons between control and single treatment conditions were made using an unpaired, two-tailed *t*-test. The assumption that data were sampled from populations with Gaussian distributions was tested using the method of Kolmogorov and Smirnov. For multiple comparisons with a common control, data were analyzed using a one-way ANOVA followed by Dunnett's test. A value of $p < 0.05$ was considered significant.

Results

The adhesion time course of individual eosinophils was determined by measuring changes in cell surface area. Images of the cells using Hoffman modulation optics were acquired at 20 s intervals for several minutes following

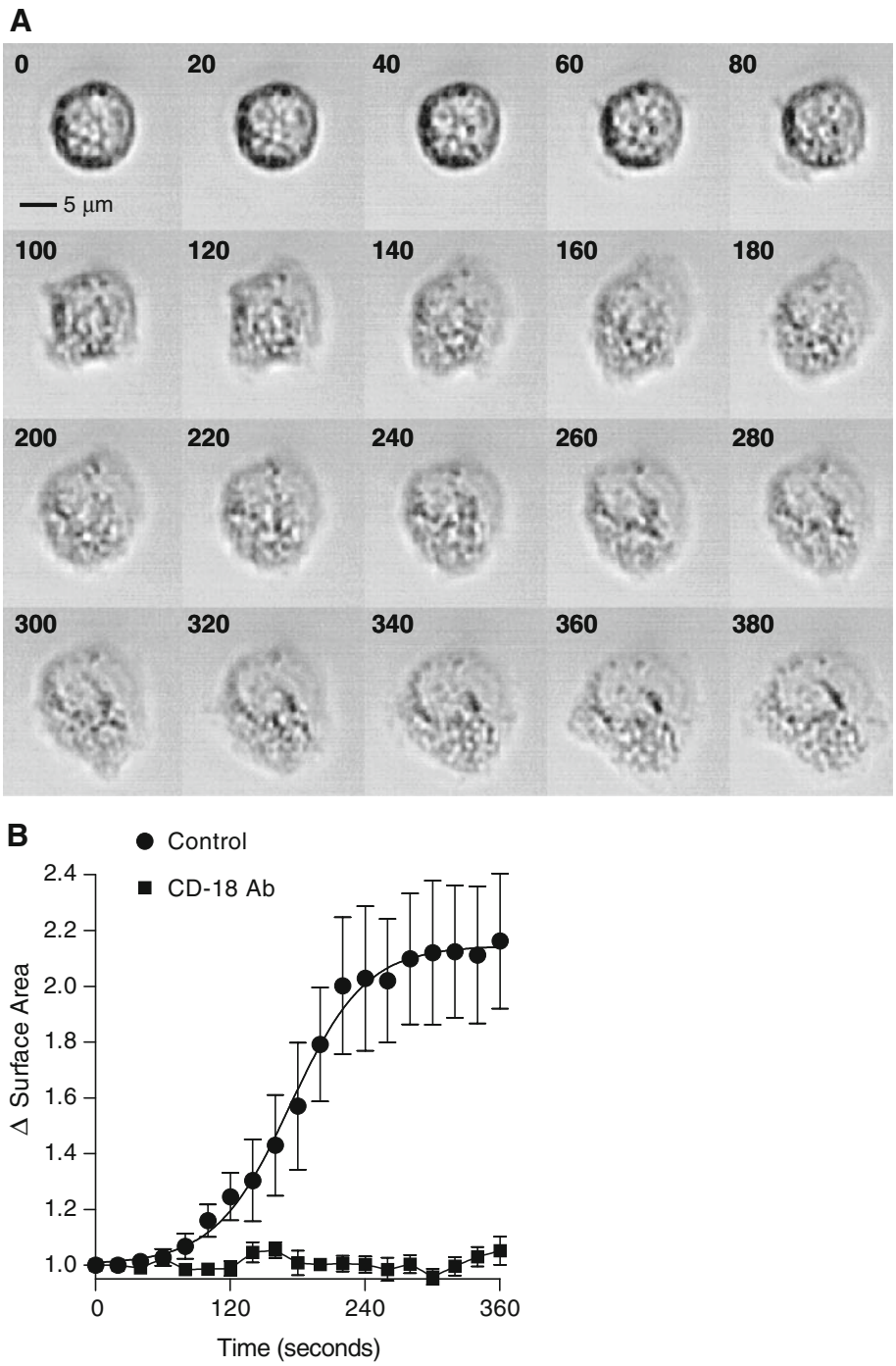

Fig. 1 **a** Images showing changes in eosinophil morphology during adhesion. The time (in seconds) after formation of the initial tethered attachment is indicated in the upper-left corner of each image. **b** Time course comparing increases in relative surface area of control eosinophils and eosinophils pretreated with CD18 blocking Ab ($n = 5$ for each condition). The initial area (mean = 80 μm^2) was normalized to a value of 1.0

plating onto glass coverslips. Figure 1 shows that the eosinophil surface area increases by approximately twofold as adhesion occurs. In addition, the transition from tethered attachment to maximum surface spreading occurs within 3 min at a temperature of 25°C. If changes in surface spreading were measured in the presence of anti-CD18 blocking antibody, the cell shape changes and surface area increase observed in control eosinophils were not observed.

Although surface area measurements effectively document changes in cell morphology associated with adhesion, image analysis is time-consuming and poorly suited for screening the effects of potential modulators of the adhesion process. To evaluate adhesion in real time in a population of eosinophils, a new method was developed that measures changes in impedance as eosinophils adhere to substrate in the presence of bovine serum albumin. Figure 2a shows the increase in impedance of control eosinophils as they spread over the electrode surface. If eosinophils are treated with anti-CD18 antibody, the increase in impedance is significantly inhibited (Fig. 2a, b).

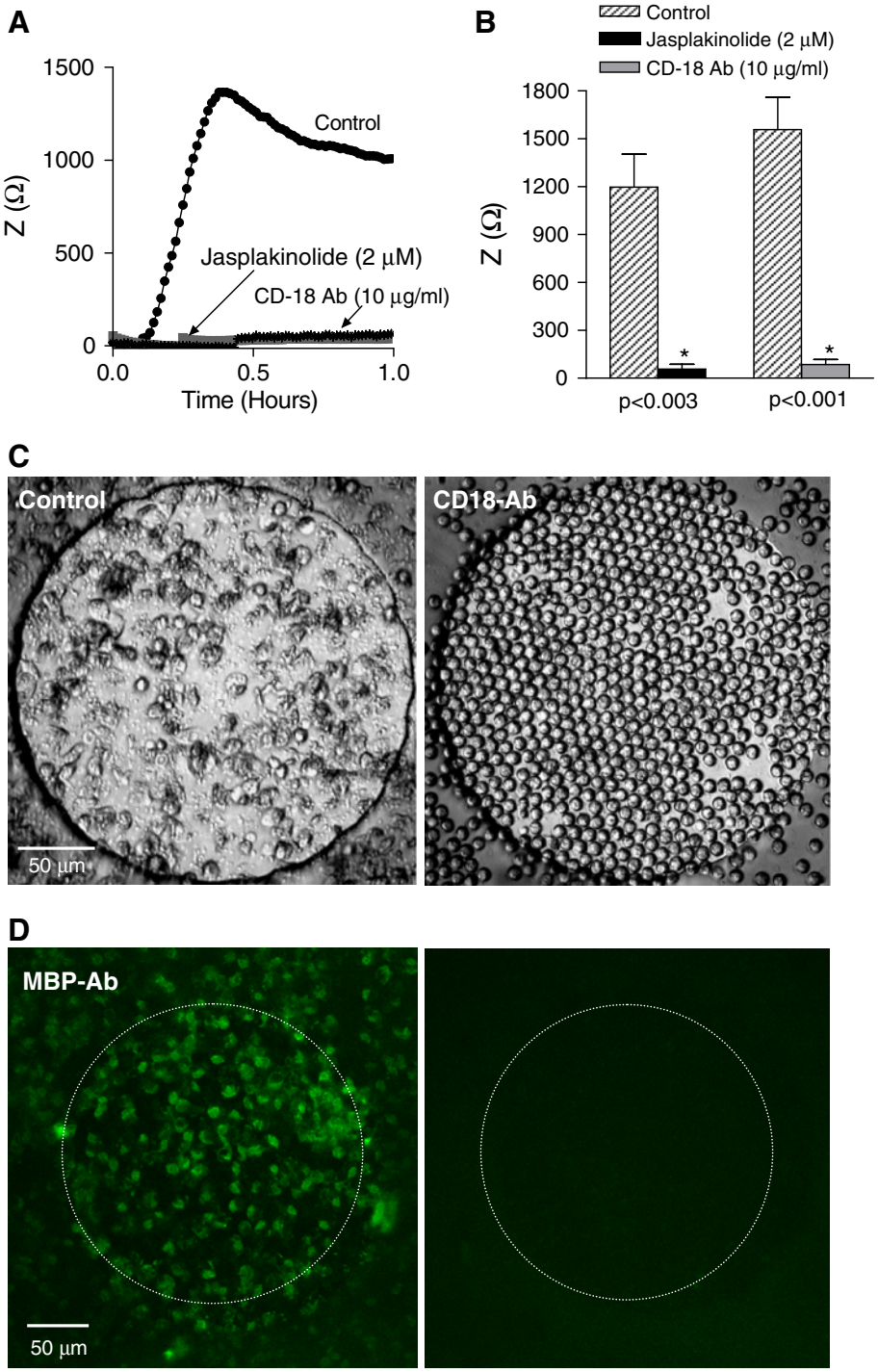

Fig. 2 **a** Time course showing changes in impedance of eosinophils under control conditions compared to cells treated with jasplakinolide (2 µM) or cells pretreated with a blocking CD18 antibody recognizing β_2-integrin. **b** Comparison of maximum impedance attained at 23 min by control eosinophils and those treated with jasplakinolide ($n = 8$) or blocking antibody ($n = 10$). **c** Cell morphology of control eosinophils at 1 h compared to cells pretreated with CD18 blocking antibody (10 µg/ml). **d** Detection of MBP release from control cells at 1 h from the beginning of the impedance measurement

Comparison of images from control and anti-CD18 antibody-treated eosinophils acquired at the end of the experiment (Fig. 2c) clearly shows that cell morphology changes associated with adhesion are apparent in control cells, but those treated with anti-CD18 blocking antibody retain a spherical morphology. The result demonstrates that increases in impedance are directly dependent on molecular interactions between the plasma membrane and the electrode surface as adhesion occurs and that tethering does not produce a detectable change in impedance. In addition, control eosinophils that have undergone adhesion begin to exhibit degranulation by the end of the experiment (1 h) as indicated by the release of major basic protein shown in Fig. 2d. Interference with normal changes in actin polymerization associated with adhesion by pretreating cells with jasplakinolide 10 min prior to the beginning of the

experiment was also shown to significantly inhibit increases in impedance similar to effects observed with anti-CD18 antibody (Fig. 2a, b).

Substrate-induced adhesion was also shown to be dependent on the activity of phosphoinositide 3-kinase (PI-3 kinase) and PKCζ (Fig. 3). Pretreatment of eosinophils with the PI-3 kinase inhibitor LY294002 (5 μM) produced significant inhibition of the impedance increase compared to control cells, but unlike with jasplakinolide or anti-CD18 blocking antibody, inhibition was not as complete. Previously reported concentration-response data have shown that the concentration of LY294002 used in these experiments was sufficient to completely inhibit superoxide formation in IL-5-activated eosinophils, therefore incomplete inhibition of adhesion was not likely to be the result of using a submaximal concentration of the compound (Bankers-Fulbright et al. 2001). A similar degree of inhibition (~70%) was observed when PKCζ activity was inhibited by pretreating cells with a membrane-permeable pseudosubstrate inhibitor.

Figure 4 shows the results of confocal imaging experiments documenting changes in eosinophil morphology and actin redistribution following treatments with jasplakinolide, LY294002, PKCζ, pseudosubstrate and anti-CD18 antibody. Representative images of eosinophils are shown in Fig. 4a. Control cells (panel 1) were allowed to make contact with the substrate and form tethered attachments (5-min exposure) prior to fixation. In panels 2–6 cells were exposed to the substrate for 30 min under the same conditions used in the impedance experiments reported in Figs. 2 and 3 to evaluate the effects of various treatment conditions on cell morphology and actin distribution. The results indicate that treatment with jasplakinolide dramatically alters actin distribution within the cell and inhibits cell spreading. This effect is further documented in Fig. 4b, which compares the fluorescence profile obtained from a transect through the diameter of the cell between control cells (forming tethered attachments) and jasplakinolide-treated cells after 30 min of contact with the substrate. Note that the maximum fluorescence intensity is relatively evenly distributed in the control cells, whereas jasplakinolide-treated cells show a redistribution of actin labeling toward the outer perimeter of the cell. An area analysis of control and all treatment conditions is presented in Fig. 4c. The results show that untreated cells undergo adhesion resulting in a twofold increase in surface area compared to control (tethered) cells. This result is very similar to the results reported in Fig. 1, where imaging using Hoffman modulation optics was used to record a twofold increase in surface area following adhesion. In contrast, treatment with LY294002, PKCζ, pseudosubstrate, or anti-CD18 antibody inhibited significant increases in cell surface area.

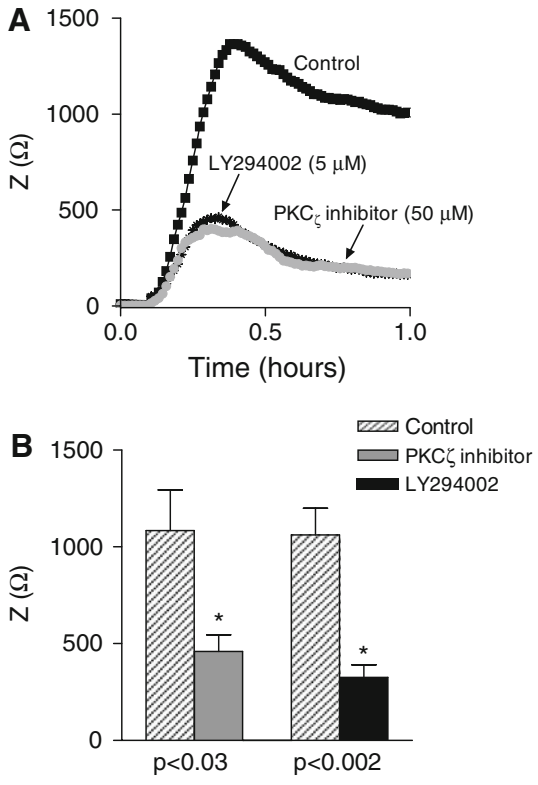

Fig. 3 **a** Time course data showing the effects of inhibiting PI-3 kinase and PKCζ on impedance changes associated with adhesion. **b** Maximum impedance attained by control eosinophils and those treated with LY294002 ($n = 6$) or PKCζ pseudosubstrate ($n = 5$)

Figure 5 shows the results of Ca^{2+} imaging experiments where simultaneous measurements of adhesion and changes in $[Ca^{2+}]_i$ were performed. For these experiments eosinophils were loaded with Fura-2 and allowed to form tethered attachments to BSA-treated glass coverslips. Cell spreading was detected (Fig. 5a) and analyzed for changes in surface area (Fig. 5b), which correlated well in both time course and magnitude with the data reported in Fig. 1. Prior to the observed changes in cell morphology, an increase $[Ca^{2+}]_i$ was recorded with the corresponding sequence of images identified in numerical order (Fig. 5b). The mean ± SE basal and peak increases in $[Ca^{2+}]_i$ are reported in reported in Fig. 5c.

Effects of Ca^{2+} mobilization on adhesion were investigated using impedance measurements and the results are presented in Fig. 6. Pretreatment of eosinophils with BAPTA-AM (10 μM) for 30 min before the start of the experiment produced a significant inhibition of the impedance increase associated with adhesion. The magnitude of the effect was similar to data shown in Fig. 3 for jasplakinolide and anti-CD18 blocking antibody. In contrast, reducing extracellular Ca^{2+} using Ca^{2+}-free medium containing 2.5 mM EGTA did not significantly inhibit increases in impedance or changes in cell spreading that occurs with adhesion. These results indicate that the

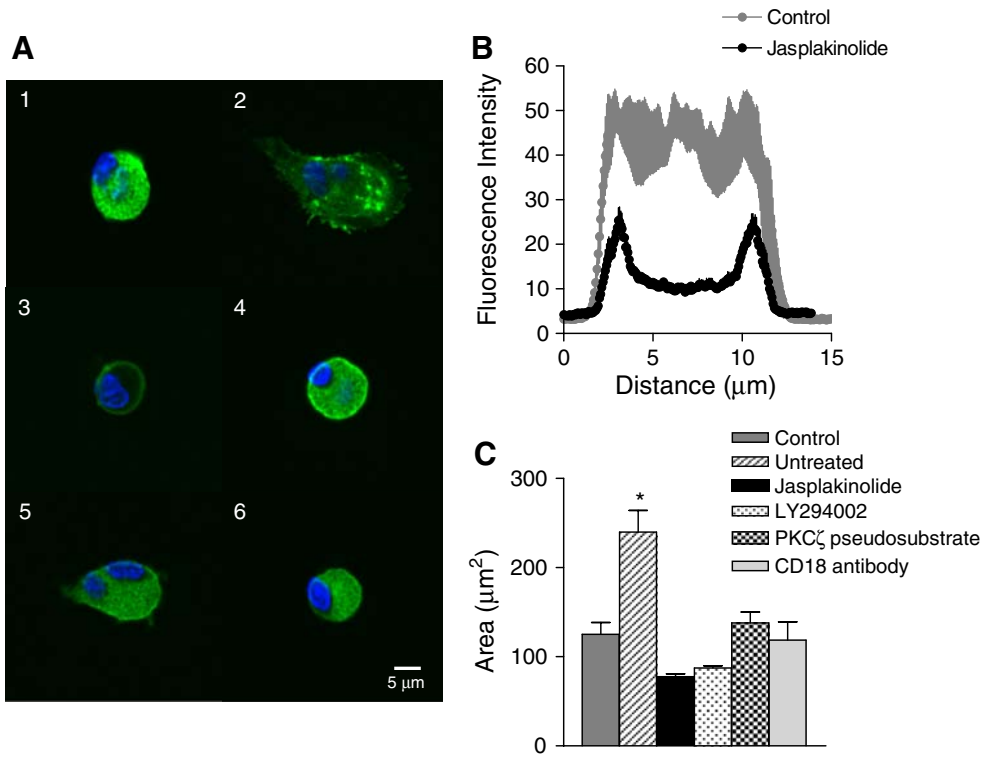

Fig. 4 **a** Representative confocal images showing actin labeling with Alexa Fluor 488-labeled phalloidin and nuclear staining with DAPI (magnification = 630×, excitation λ/emission filter λ = 488/505–550 and 364/385–470 nm for Alexa Fluor 488 and DAPI, respectively). Panel 1: control cells (tethered attachment). Panel 2: untreated cells after 30 min of substrate contact. Panel 3: jasplakinolide (2 μM) treatment during the 30-min exposure to substrate. Panel 4: LY294002 (5 μM) treatment during the 30-min exposure to substrate. Panel 5: PKCζ pseudosubstrate inhibitor (50 μM) treatment during the 30-min exposure to substrate. Panel 6: anti-CD18 antibody (12.5 μg/ml) treatment during the 30-min exposure to substrate. **b** Fluorescence profile comparing relative fluorescence intensity obtained from transects across the diameter of control cells to those exposed to 2 μM jasplakinolide. Values represent the mean ± SE for 12 cells for each condition. Profiles were generated using Image J software (NIH). **c** Area measurements (mean ± SE) for each condition listed in **a** (n = 12). Areas were measured using Zeiss LSM Image Browser software

increase in cell Ca^{2+} is due to mobilization from intracellular stores and not directly dependent on Ca^{2+} influx from the extracellular solution.

Discussion

The conventional model for leukocyte adhesion consists of three steps that include selectin-mediated rolling, chemokine-dependent activation, and integrin-regulated arrest. Additional steps have been identified more recently that include slow rolling, adhesion strengthening, intraluminal crawling, paracellular migration, and migration across the basement membrane (Ley et al. 2007). Granulocyte arrest appears to be triggered by chemokines or other locally produced inflammatory mediators and is dependent on binding to adhesion molecules such as ICAM-1 and VCAM-1 expressed by endothelial or epithelial cells. For eosinophils, initial attachment to activated endothelial cells involves primary tethering that is predominantly mediated by P-selectin (Kitayama et al. 1997). The integrins involved in arrest are members of the β_1 (VLA4)- and β_2 (CD11b/CD18 or LFA1)-integrin subfamilies, where chemokine binding to various receptors increases both surface expression and avidity. Subsequent adhesion strengthening is dependent on the ability of integrins to produce intracellular signals that regulate a variety of cell functions. Integrin-dependent signal transduction is often referred to as outside-in signaling and studies with eosinophils have shown that pathways involving PI-3 kinase, p38 MAP kinase, and PKC are critical for adhesion (Ley et al. 2007).

In the present study eosinophil adhesion was investigated in vitro under conditions where cells were allowed to interact with substrate, but in the absence of exogenously applied signaling molecules. Adhesion was monitored in individual eosinophils by measuring changes in surface area contact with the substrate and in populations of eosinophils using a new method that measures increases in impedance associated with the formation of β_2-integrin-dependent attachment to the substrate. Eosinophils were shown to undergo spreading that was dependent on actin polymerization and inhibited by anti-CD18 blocking

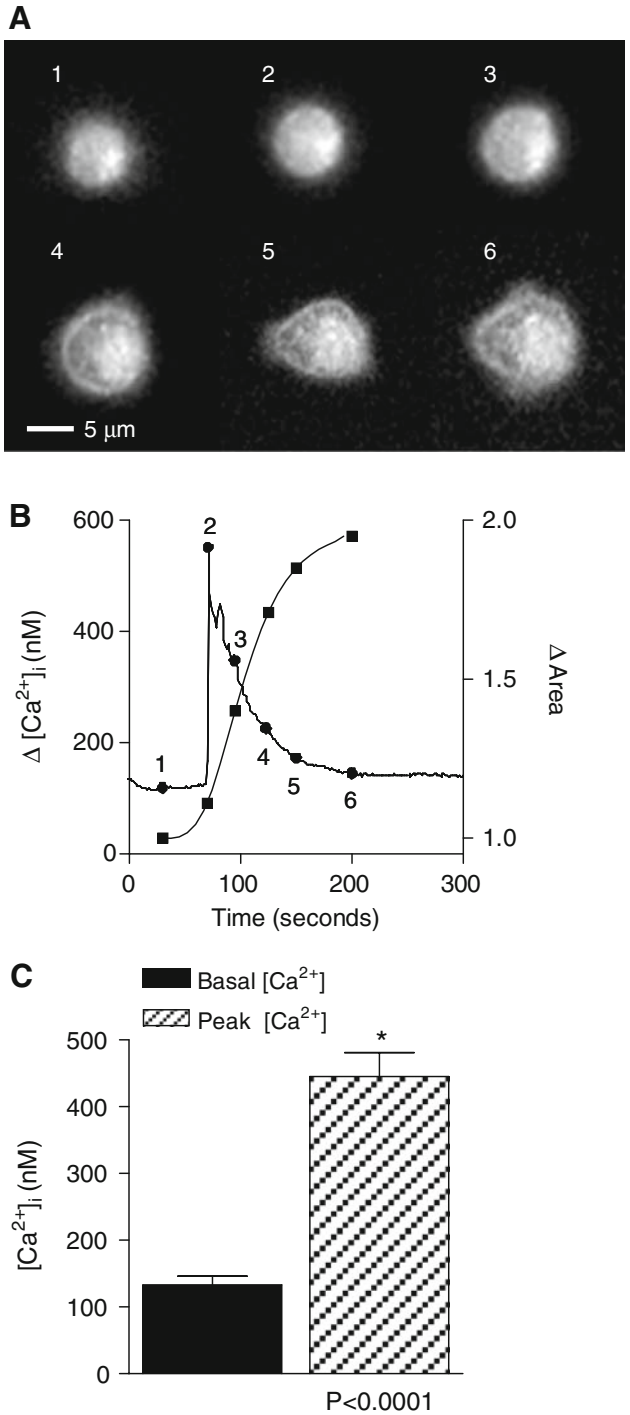

Fig. 5 **a** Changes in cell morphology measured simultaneously with changes in $[Ca^{2+}]_i$. **b** Time course showing the change in relative surface area and the transient increase in $[Ca^{2+}]_i$ in eosinophils prior to cell spreading. The numbered points indicated in the tracing correspond to the images showing cell morphology changes during adhesion (in **a**). **c** Basal and peak $[Ca^{2+}]_i$ measured in cells undergoing adhesion ($n = 7$)

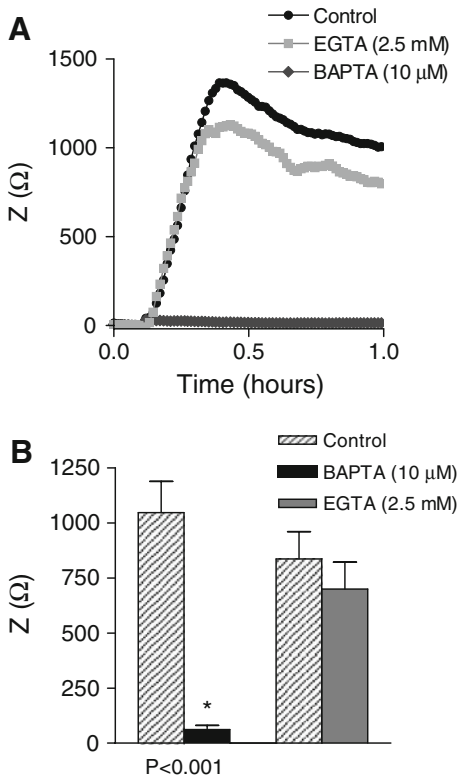

Fig. 6 **a** Time course data showing effects of chelating intracellular Ca^{2+} with BAPTA-AM or lowering extracellular Ca^{2+} in Ca^{2+}-free medium with 2.5 mM EGTA on changes in impedance. **b** Measurements of peak impedance values from control and BAPTA-AM ($n = 6$)- or EGTA ($n = 5$)-treated eosinophils

antibodies. Confocal imaging confirmed that blocking β_2-integrin interactions with the substrate inhibits actin reorganization and associated morphologic changes that occur with adhesion. The time course of changes in surface area contact with the substrate indicated that, once initiated, eosinophil spreading reached its maximum within 5 min. In contrast, maximum impedance was observed in ~14 min, indicating that additional time beyond that needed for cell spreading was necessary for eosinophils to fully adhere to the substrate. We speculate that this difference was due to a slower time course for additional β_2-integrin recruitment, clustering, and activation on the cell surface compared to actin polymerization and membrane protrusion events that occur with spreading. Interestingly, a gradual decrease in impedance was detected after the peak. Inspection of eosinophil morphology and MBP staining at the end of the experiment indicated that the eosinophils had undergone degranulation, which is associated with loss of plasma membrane integrity. Thus the decrease in impedance most likely reflects cell damage that occurs following eosinophil activation and degranulation.

The increase in impedance was also partially blocked (~60%) by LY294002, an inhibitor of PI-3 kinase, a result that was previously reported for eosinophil adhesion (Bankers-Fulbright et al. 2001; Kato et al. 2005a; Sano et al. 2005; Schaff et al. 2008; Trucy et al. 2006). A similar

level of inhibition (~50%) was also reported with the PI-3 kinase inhibitor wortmanin using Calcein-AM fluorescence to measure eosinophil adhesion (Lynch et al. 1999). The results also showed that adhesion was blocked to the same extent as observed with LY294002 following incubation of cells with PKCζ pseudosubstrate. This finding was consistent with a previous study showing a role for this atypical PKC isoform in β_2-integrin-regulated adhesion in granulocytes (Kato et al. 2005a; Yamaguchi et al. 2006).

The results of this study and findings from previously published reports suggest that outside-in signaling responsible for adhesion strengthening/stabilization occurs after the Ca^{2+} mobilization step and involves activation of PI-3 kinase. The mechanism of PI-3 kinase activation has not been definitively established in eosinophils, but previous studies have demonstrated that the enzyme can be stimulated by increases in $[Ca^{2+}]_i$ (Liu et al. 2007) or by tyrosine phosphorylation (Kim et al. 1995). PI-3 kinase activation results in increased synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) and phosphatidyl 3,4-bisphosphate. Activation of PKCζ depends on PIP3, and 3′-PI-dependent protein kinase 1, which binds with a high affinity to PIP3, phosphorylates PKCζ, and activates the enzyme. Thus PKCζ is part of a signaling cascade that depends on PI-3 kinase, which may explain why blocking either of these enzymes results in a similar magnitude of adhesion inhibition (Frey et al. 2006; Liu et al. 2007; Martin et al. 2001; Rojnuckarin et al. 2001; Trucy et al. 2006). The reason why adhesion is not completely blocked by PI-3 kinase or PKCζ inhibition is not clear. In a previous study investigating the mechanism of IL-5-induced adhesion in eosinophils, it was demonstrated that Ras activation serves as a parallel mechanism to increase β_2-mediated adhesion and that inhibition of PI-3K or PKCζ does not appear to disrupt this pathway (Myou et al. 2002; Sano et al. 2005). Perhaps a similar role for Ras is responsible for the PI-3K/PKCζ-independent adhesion observed in the present study.

A recent study of neutrophil recruitment under flow conditions demonstrated that increases in $[Ca^{2+}]_i$ represent an early and essential phase of signaling that links selectin and chemokine receptor ligation to adhesion and subsequent cell migration (Schaff et al. 2008). Integrin-mediated neutrophil arrest initiated by E-selectin tethering and IL-8 binding to its receptor was shown to be preceded by a rise in $[Ca^{2+}]_i$. Interestingly, inhibition of Ca^{2+} influx through store-operated Ca^{2+} channels inhibited β_2-integrin activation and corresponding neutrophil arrest. It was concluded from this study that Ca^{2+} influx integrates chemotactic and adhesive signals in a manner that synchronizes signaling of neutrophil arrest and migration. Results of the present study also demonstrated that a critical initiating event for β_2-integrin-mediated adhesion in human eosinophils is an increase in intracellular $[Ca^{2+}]$ and that preventing the increase completely inhibited adhesion. However, lowering extracellular $[Ca^{2+}]$ as a means to decrease the driving force for Ca^{2+} entry did not significantly reduce adhesion or alter the time course of impedance changes associated with the process. Thus eosinophils exhibit a similar dependence on $[Ca^{2+}]_i$ for initiating adhesion as described in neutrophils, but Ca^{2+} influx through store-operated Ca^{2+} channels does not appear to be required. This is not to say that these channels are not present in eosinophils or activated in response to store depletion, but only that Ca^{2+} mobilization from internal stores is sufficient for spreading and adhesion to proceed.

A model summarizing the findings of this study is shown in Fig. 7. Following formation of a tethered attachment to the substrate, we speculate that β_2-integrin engagement initiates tyrosine kinase-mediated phosphorylation of Cbl and subsequent activation of PLCγ to produce IP_3 and mobilization of Ca^{2+} from internal stores. Calcium release facilitates integrin recruitment/clustering on the plasma membrane and actin polymerization necessary for cell spreading. In addition, activation of PI-3 kinase and PKCζ appears to participate in spreading by facilitating actin reorganization and integrin engagement as suggested by increases in impedance. Although the precise mechanism by which PI-3 kinase and PKCζ affect adhesion is not completely understood, previous studies have shown that

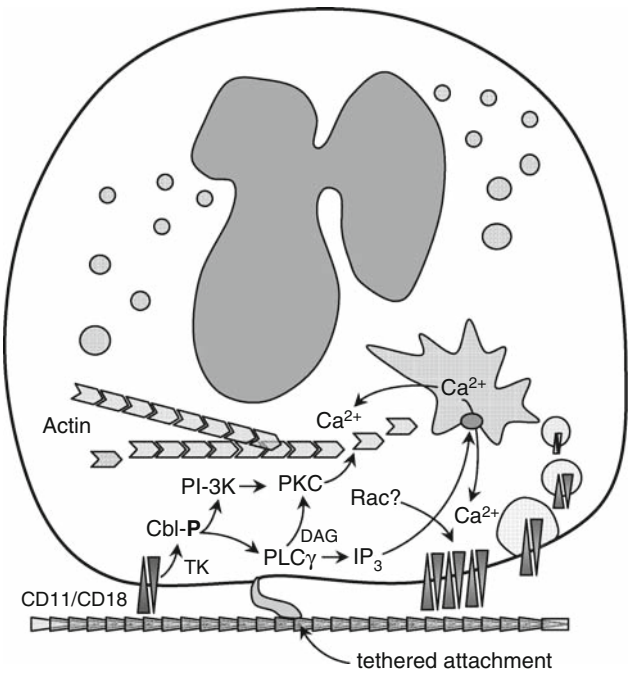

Fig. 7 A proposed cell model explaining the results of this study. See Discussion for details

ERK phosphorylation and cytosolic phospholipase A_2 activation are critical steps (Trucy et al. 2006; Zhu et al. 2002). Ras activation leading to focal clustering and activation of CD11/CD18 has also been implicated in IL-5-, eotaxin-1-, and fMLP-induced adhesion and may also play a similar role in β_2-integrin-initiated adhesion.

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