Elevation of Intracellular cAMP Inhibits RhoA Activation and Integrin-dependent Leukocyte Adhesion Induced by Chemoattractants

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Chemoattractant receptors of the serpentine, heterotrimeric G protein-linked family can activate leukocyte integrins and in this role regulate leukocyte traffic and cell-cell interactions in immune and inflammatory responses. Using a mouse lymphoid cell line transfected with human formyl peptide or interleukin-8 receptors and normal human neutrophils as models, we show that cAMP functions as a gating element on the chemoattractant-induced rho-dependent signaling pathway leading to leukocyte integrin activation and adhesion. cAMP, acting through protein kinase A, inhibits chemoattractant-triggered integrin-dependent leukocyte adhesion. cAMP also prevents guanine nucleotide exchange on RhoA, a small GTP-binding protein of the rho subfamily, which is activated in seconds by chemoattractants. In contrast, chemoattractant-triggered intracellular calcium elevation is unaffected by cAMP, and cAMP has no effect on rho-dependent adhesion and RhoA guanine nucleotide exchange triggered through the independent protein kinase C pathway. These data suggest that cAMP-induced inhibition of rho activation may be responsible for the anti-adhesive effect of cAMP and may contribute to the anti-inflammatory activity of cAMP elevating agonists and drugs. Moreover, the findings extend the concept of cyclic nucleotide gating as a broadly important mechanism in the regulation of intra- cellular signaling pathways and the cellular activities they control.

The regulation of integrin-dependent adhesion and de-adhesion is important in leukocyte cell-cell and cell-matrix interactions in immunity and inflammation. Serpentine receptors of the Gαi-linked chemoattractant receptor subfamily have been implicated in leukocyte adhesion regulation and are thought to play essential roles in controlling leukocyte trafficking and homing in vivo. These receptors stimulate an amplified and branching cascade of second messengers triggered through either α or βγ subunits of heterotrimeric GTP-binding proteins (1). The small GTP-binding protein rho has recently been identified as a critical element in the signaling cascade responsible for fast integrin-dependent leukocyte adhesion (2). Chemoattractants stimulate very rapid guanine nucleotide exchange on the small G-protein RhoA, and inhibition of rho by C3 transferase inhibits agonist-triggered integrin activation. The pathway linking chemoattractant receptors to rho activation is still unknown but seems to be independent of diacylglycerol (DAG)1-dependent protein kinase C (PKC) (2).

To explore further the regulation of chemoattractant to integrin signaling, we have assessed the effect of cAMP, a potent inhibitor of several leukocyte proinflammatory activities such as NADPH oxidase activation, granule exocytosis in neutrophils, and leukocyte transendothelial migration (3–5). We report that intracellular cAMP, acting through protein kinase A (PKA), abrogates the proadhesive response of lymphoid cells and of neutrophils to chemoattractant but not to phorbol ester stimulation. This inhibitory effect is associated with blockade of chemoattractant-induced guanine nucleotide exchange on the small GTP-binding protein RhoA, suggesting that cAMP-dependent PKA acts as a negative modulator or “gate” on the chemoattractant to rho to integrin signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—PBS, fMLP, PMA, Bt,cAMP, theophylline, control rabbit antibody to mouse immunoglobulin, Triton X-100, deoxycholate, SDS, benzamidine, leupeptin, pepstatin, aprotinin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, EGTA, EDTA, dithiothreitol, GTP, GDP, and human fibrinogen were purchased from Sigma; fetal calf serum (FCS), RPMI 1640, phosphate-free RPMI 1640, and dialyzed FCS were purchased from Irvine; 32Porthophosphate was from Amersham Corp.; rabbit polyclonal anti-RhoA, which recognizes the sequence KDLRNDEHTRRELA, was from Santa Cruz Biochemicals; rabbit polyclonal anti-RhoA, which recognizes the sequence KDLRNDEHTRRELA, was from Santa Cruz Biochemicals; Trasylol was purchased from Bayer; antiserum to mouse immunoglobulin, antibody to mouse immunoglobulin, Triton X-100, deoxycholate, SDS, and NIH-Image 1.56 as cell-counting software. Background binding in

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The abbreviations used are: DAG, diacylglycerol; PKC, protein kinase C; PKA, protein kinase A; PBS, phosphate-buffered saline; fMLP, formylmethionyleucylphenylalanine; PMA, phorbol 12-myristate 13-acetate; IL, interleukin; Vascular cell adhesion molecule-1 (VCAM-1), vascular cell adhesion molecule-1; GTP, guanosine nucleotide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; BSA, bovine serum albumin; GTPγS, guanosine 5′-3′-O-thiotriphosphate; RA, receptor A.

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FIG. 1. B_{2}cAMP inhibits chemoattractant-induced lymphocyte adhesion to VCAM-1 in a PKA-dependent manner. A, effect of B_{2}cAMP (dBcAMP) on chemoattractant-induced lymphocyte adhesion to mouse mVCAM-1. Human P9 or human IL-8RA transfectants were treated with Me_{6}SO (control) or with the indicated concentrations of B_{2}cAMP (Sigma) + 1 mM theophylline (Sigma) for 20 min at 37 °C in RPMI 1640. FMLP-, IL-8-, or PMA-induced adhesion to VCAM-1-coated wells was assessed. B, effect of PKA inhibitors on B_{2}cAMP-dependent inhibition of lymphocyte adhesion. Human P9 or human IL-8RA transfectants were treated for 20 min at 37 °C with Me_{6}SO (control) or 200 mM H89 (L. C. Laboratories, Woburn, MA) or 5 μM HA1004 (L. C. Laboratories, Woburn, MA) prior to treatment with 200 μM B_{2}cAMP in the presence of 1 mM theophylline. FMLP- or IL-8-induced adhesion to VCAM-1-coated wells was assessed. Values are the mean counts of bound cells in three experiments, presented with standard deviations. Background binding in the absence of agonist was minimal (less than 2% of stimulated control) and was subtracted.

The absence of added agonist was determined for each condition, was minimal (less than 2% of stimulated adhesion), and was subtracted from agonist-stimulated adhesion for data presentation.

Adhesion Assay with Human Polymorphonuclear Neutrophils (PMNs)—Human PMNs were isolated from venous blood from healthy adult volunteers as previously reported (5). The entire isolation procedure was conducted at 4 °C, using lipopolysaccharide-free medium. Whole glass slides were coated for 60 min at 37 °C with 10 μg human fibrinogen in lipopolysaccharide-free water. 5 × 10^{6} cells (0.5 ml of the suspension) were added to the coated glass, incubated for 10 min at 37 °C, and then stimulated by addition of the agonists for 1 min (100 nM fMLP or 100 ng/ml IL-8) or 10 min (100 ng/ml IL-8). After the treatment the PMNs were washed and resuspended in RPMI 1640. Background (no agonist) adhesion was 51 ± 7 cells/0.2 mm² and was subtracted.

Inhibition of Up-regulation of Neutrophil β₂-integrin Expression by 4,4′-Disothiocyanostilbene-2,2′-disulfonic Acid (DIDS)—Human PMNs (2.5 × 10^{6} cells in RPMI 1640, 20 μg HEPES, pH 7.3) were pretreated with the indicated concentrations of DIDS (Sigma) for 20 min at 37 °C with 250 mM DIDS (Sigma). The cells were then used in adhesion assay as described above. Alternatively, β₂-integrin expression was evaluated by fluorescence-activated cell sorter in buffer (control) or DIDS-treated PMNs, stimulated at 37 °C with 100 nM fMLP for 1 min, and stained with the anti-CD18 mouse monoclonal antibody IC4, as described (8).

Measurement of Guanine Nucleotide Accumulation on RhoA—L1/2 cell transfectants were incubated overnight at 37 °C in phosphate-free RPMI 1640, 10% dialyzed FCS and labeled with 0.2 μCi/ml [35S]orthophosphate for 4 h in the same medium. Cells were resuspended at 4 × 10^{6} cells/ml in PBS, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml BSA and stimulated with the appropriate agonists at 37 °C while stirring. 2 × 10^{6} cells (0.5 ml of the suspension) were lysed on ice in 0.5 ml of 100 mM HEPES buffer, pH 7.4, 2% Triton X-100, 1% deoxycholate, 0.1% SDS, 300 mM NaCl, 10 mM MgCl₂, 2 mM EGTA, 2 mg/ml BSA, 20 mM benzamidine, 20 μg/ml leupeptin-pestatin-aprotinin-soybean trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride. Nuclei were pelleted and lysates were adjusted to 500 mM NaCl. After precleaving, the samples were immunoprecipitated with 2 μg of rabbit anti-RhoA polyclonal antibody recognizing the sequence KDLRNDEHTTRELA (119–132) or rabbit anti-mouse Ig negative control for 60 min at 4 °C, followed by 4 μl of Triaacryl-protein A beads for 90 min. The beads were washed 10 times in 50 mM HEPES buffer, pH 7.4, 500 mM NaCl, 0.1% Triton X-100, 0.005% SDS, and the nucleotides were eluted in 5 ml EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP for 30 min at 68 °C (9). Separation of eluted nucleotides was on polyethyleneimine cellulose plates run in 0.75 m KH₂PO₄, pH 3.5, as described (9). Radioactive spots, determined by autoradiography with X-Omat AR films (Kodak), were scraped off the plates and counted in a scintillation β-counter. Alternatively, the cells were resuspended at 5 × 10^{7} cells in Ca²⁺/Mg²⁺-free PBS, 1% pluronic F-68 (Sigma), and 60 μCi/ml GTPγS. The cells were syringe-loaded through a tuberculin syringe with a 30-gauge needle (14). After 14 passes through the needle, 0.5–1% of added radioactive activity was incorporated into the cells. The cells were washed twice, resuspended at 4 × 10^{7} cells/ml in PBS, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml BSA, and after 10 min of recovery at 37 °C, stimulated with the appropriate agonists at 37 °C while stirring and processed as above. Radioactivity was detected with a Molecular Dynamics PhosphorImager 445 SI after 2 days of exposure.

RESULTS

cAMP Inhibits Chemoattractant-induced Integrin-dependent Lymphocyte Adhesion—To study the intracellular regulation of chemoattractant-induced lymphocyte adhesion and rho activation, we have used mouse lymphoid L1/2 cells transfected with human (IPR) or with IL-8RA as a model. Agonist stimulation of these cells induces rapid adhesion to VCAM-1. Triggered binding occurs within seconds and is mediated by activation of the integrin α₅β₁ (CD49d/CD29) (6, 7).

The second messenger cAMP regulates a number of signal transduction pathways (10). To evaluate the effect of cAMP on rapid chemoattractant-triggered adhesion, we pretreated L1/2 transfectants with B_{2}cAMP, a permeable analog of cAMP. B_{2}cAMP treatment inhibited IL-8 or fMLP-induced adhesion in a dose-dependent manner. In contrast, adhesion induced by the phorbol ester PMA, an activator of DAG-dependent PKCs, was not affected by cAMP pretreatment (Fig. 1A). The inhibitory effect of B_{2}cAMP was not due to metabolic release of butyrate or to contamination of B_{2}cAMP with butyrate because butyrate itself (200 μM) had no effect on binding (see control data in Fig. 1B). The most prominent effector of cAMP is PKA. Pretreatment with specific PKA inhibitors, H89 or HA1004, blocked the inhibitory effect of B_{2}cAMP, completely restoring agonist-induced VCAM-1 binding in response to fMLP and IL-8 (Fig. 1B). We conclude that cAMP through its effector PKA inhibits chemoattractant activation of the lymphocyte integrin α₅β₁.

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recently reported (2, 14). Transfected L1/2 cells were labeled with [32P]orthophosphate (0.2 mCi/ml for 4 h) and resuspended at 4 × 10^7/ml in PBS, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml BSA, were preincubated for 30 min at 37 °C with buffer control or with the indicated amount of Bt₂cAMP in the presence of 1 mM theophylline. Agonist stimulation was for 1 min at 37 °C while stirring. The radioactivity bound to immunoprecipitated RhoA migrates with the GDP standard. Bt₂cAMP pretreatment on chemoattractant-induced accumulation of GTPγS on RhoA 100 nM fMLP, 100 ng/ml IL-8, and 150 ng/ml PMA. Transfected cells, loaded with GTPγS and resuspended at 4 × 10^7/ml in PBS, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml BSA, were preincubated for 30 min at 37 °C with buffer or with 400 μM Bt₂cAMP in the presence of 1 mM theophylline. Agonist stimulation was for 1 min (fMLP and IL-8) or 5 min (PMA) at 37 °C while stirring. C. Bt₂cAMP treatment has no effect on the total amount of RhoA immunoprecipitated. The figure illustrates an anti-RhoA probed Western blot of anti-RhoA precipitates from lysates of fMLP-stimulated transfectants (1 min at 37 °C, 100 μM) pretreated with buffer (control, left lane) or with Bt₂cAMP (400 μM in the presence of 1 mM theophylline, as above, right lane).

PKA Regulation of Chemoattractant-induced Rho-dependent Adhesion

FIG. 3. cAMP inhibits chemoattractant-stimulated guanine nucleotide exchange on RhoA. A, effect of Bt₂cAMP (dBcAMP) pre-treatment on chemoattractant-induced radioactive GDP accumulation on RhoA 100 nM fMLP and 100 ng/ml IL-8. Transfected cells, labeled with [32P]orthophosphate (0.2 mCi/ml for 4 h) and resuspended at 4 × 10^7/ml in PBS, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml BSA, were preincubated for 20 min at 37 °C with buffer (control) or with the indicated amount of Bt₂cAMP in the presence of 1 mM theophylline. Agonist stimulation was for 1 min at 37 °C while stirring. The radioactivity bound to immunoprecipitated RhoA migrates with the GDP standard. Bt₂cAMP pretreatment on chemoattractant-induced accumulation of GTPγS on RhoA 100 nM fMLP, 100 ng/ml IL-8, and 150 ng/ml PMA. Transfected cells, loaded with GTPγS and resuspended at 4 × 10^7/ml in PBS, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml BSA, were preincubated for 30 min at 37 °C with buffer or with 400 μM Bt₂cAMP in the presence of 1 mM theophylline. Agonist stimulation was for 1 min (fMLP and IL-8) or 5 min (PMA) at 37 °C while stirring. C. Bt₂cAMP treatment has no effect on the total amount of RhoA immunoprecipitated. The figure illustrates an anti-RhoA probed Western blot of anti-RhoA precipitates from lysates of fMLP-stimulated transfectants (1 min at 37 °C, 100 μM) pretreated with buffer (control, left lane) or with Bt₂cAMP (400 μM in the presence of 1 mM theophylline, as above, right lane).

PKA Inhibits Chemoattractant-stimulated Guanine Nucleotide Exchange on RhoA in Lymphocytes—Previous studies have shown that the small GTP-binding protein rho is an important intracellular mediator of integrin triggering both through chemoattractant receptors (2) and also through PMA-activated PKC (2, 12). The ability of cAMP to inhibit chemoattractant but not PMA-induced leukocyte adhesion (Figs. 1A and 2) suggested that PKA might act upstream of rho, blocking a mechanism of rho activation specifically triggered by G-protein-linked chemoattractant receptors. On the other hand, Bt₂cAMP has no effect on fMLP- or IL-8-triggered elevation in intracellular calcium in transfected or in neutrophils (data not shown); thus it does not inactivate the chemoattractant receptor itself. To test the effect of elevation of intracellular cAMP on chemoattractant-induced rho activation, we evaluated guanine nucleotide exchange on RhoA, the predominant rho protein in lymphocytes (12). Rho small G-proteins have high intrinsic GTPase activity so that GDP/GTP exchange on RhoA is followed rapidly by conversion of bound GTP to GDP. This rapid hydrolysis precludes detection of their GDP-bound form in vivo (13); we therefore assessed accumulation of 32P-labeled GDP on immunoprecipitated RhoA as a measurement of stimulated rho guanine nucleotide exchange activity, as previously reported (2, 14). Transfected L1/2 cells were labeled with radioactive phosphate, and the accumulation of 32P-labeled GDP was measured. As shown in Fig. 3A, the amount of 32P-labeled GDP bound to RhoA, which is very low in resting cells, was increased 6–8-fold by stimulation with fMLP or IL-8, as reported previously (2). Pretreatment of leukocytes with Bt₂cAMP inhibited agonist-induced accumulation of 32P-labeled GDP on RhoA in a dose-dependent manner, up to 85% for fMLP or 83% for IL-8. To confirm this finding, transfected L1/2 cells were loaded with GTPγS, an hydrolysis-resistant radioactive analog of GTP (2). As shown in Fig. 3B, stimulation of cells with either fMLP, IL-8, or PMA triggered binding of GTPγS to RhoA. In contrast RhoA did not bind GTPγS in non-stimulated cells, as previously reported (2). Pretreatment of leukocytes with Bt₂cAMP inhibited fMLP and IL-8-induced accumulation of GTPγS on RhoA. However, PMA-induced accumulation of GTPγS on RhoA was unaffected. Importantly, Bt₂cAMP treatment had no effect on the quantity of RhoA protein immunoprecipitated from stimulated cells (Fig. 3C), implying that the reduction of 32P-labeled GDP or GTPγS bound to RhoA in Bt₂cAMP-treated cells is due to a decrease of RhoA guanine nucleotide exchange activity. Thus,

![Diagram](image)

Fig. 4. Schematic summary of the proposed function of cAMP as a gating molecule in chemoattractant-triggered integrin activation and leukocyte adhesion. Pertussis toxin-sensitive G protein-linked chemoattractant receptors activate rho GDP/GTP exchange activity. Rho-GTP triggers downstream signals leading to integrin activation and leukocyte adhesion. CAMP inhibits both rho GDP/GTP exchange activity and integrin activation through chemoattractant receptors. DAG-dependent PKC can activate rho and adhesion as well, but this pathway is not required for rapid adhesion triggered by chemoattractants and is not gated by cAMP.
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cAMP inhibits chemoattractant-induced rapid activation of RhoA.

**DISCUSSION**

We have shown that elevation of intracellular levels of cAMP blocks chemoattractant stimulation of $\alpha_\beta_1$-integrin activation in lymphoid cells, and $\beta_2$-integrin triggering in neutrophils. The effect is mediated by PKA, and this PKA-dependent inhibition occurs downstream of heterotrimeric G-protein activation but upstream of the small GTPase RhoA, a critical mediator of chemoattractant to integrin signaling (2). Recent studies have highlighted the importance of intracellular cAMP as a gating element in different signaling pathways (10), including mitogen-activated protein kinase activation and cellular proliferation stimulated through growth factor receptors (24–26), and long range patternning during development mediated by the diffusible morphogen Sonic Hedgehog (27). Our results expand this concept to include cAMP and its effector, PKA, as gating elements in chemoattractant stimulation of rho and of rho-dependent integrin activity leading to leukocyte adhesion.

An independent example of the negative role of cAMP on rho-dependent signaling has been previously suggested. In a study of human NK cells, cAMP inhibited spontaneous rho-dependent slow cell movement. In that model, PKA phosphorylation of active (GTP-bound) RhoA induced gradual rho-guanine dissociation inhibitor to bind rho. Moreover, our data show rho-signaling pathway, increasing the capability of rho-guanine exchange by cAMP, reported here, which allows cAMP to prevent inhibition of chemoattractant-stimulated RhoA GDP/GTP exchange by cAMP, reported here, which allows cAMP to prevent the initiation of rho signaling, thus blocking the rapid rho-dependent triggering of integrins by chemoattractants. Thus, it appears that cAMP can be a negative modulator of rho through two separate mechanisms, either by preventing rapid rho activation, as shown here, or by terminating an already active rho-signaling pathway, increasing the capability of rho-guanine dissociation inhibitor to bind rho. Moreover, our data show for the first time that cAMP can inhibit a small GTP-binding protein-dependent pathway by blocking the activation of the GTPase itself (Fig. 4).

In addition to triggering integrin activation rho mediates cytoskeletal remodeling (16), and in both of those roles it is thought to be important to cell trafficking. The inhibitory activity of PKA on rho activation in leukocytes may thus help explain the ability of some cAMP-elevating drugs to inhibit leukocyte transendothelial migration in vitro and recruitment and homing in vivo (17–21), phenomena that are dependent on chemoattractants and integrins. The effect may also permit cross-talk between pro-adhesive and anti-adhesive heterotrimeric G protein-linked receptors, potentially contributing, for example, to the inhibition of chemoattractant-induced neutrophil migration by adenosine, prostaglandin E1, or $\beta_2$-adrenergic receptors (22, 23), Go$_\alpha$-linked serpentine receptors that activate adenyl cyclase to produce cAMP.

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