Immune Complex-induced Integrin Activation and L-plastin Phosphorylation Require Protein Kinase A*

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Integrins in resting leukocytes are poorly adhesive, and cell activation is required to induce integrin-mediated adhesion. We recently demonstrated a close correlation between phosphorylation of Ser5 in L-plastin (LPL), a leukocyte-specific 67-kDa actin bundling protein, and activation of α5β2-mediated adhesion in polymorphonuclear neutrophils (PMN) (Jones, S. L., Wang, J., Turck, C. W., and Brown, E. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9331–9336). However, the kinase that phosphorylates LPL Ser5 has not been identified. We found that cAMP-dependent protein kinase (PKA), but not a variety of other serine kinases, can specifically phosphorylate LPL and LPL-derived peptides on Ser5 in vitro. The cell-permeable cAMP analog 8-bromo-cAMP and the adenylate cyclase activator forskolin both induce LPL phosphorylation in cells. Two PKA inhibitors, H89 and KT5720, inhibited immune complex (IC)-stimulated LPL phosphorylation as well as IC-induced activation of α5β2-mediated adhesion in PMN. The dose response of H89 inhibition of PMN adhesion correlated with its inhibition of LPL phosphorylation in response to IC. IC stimulation also transiently increased intracellular cAMP concentration in PMN. Thus, PKA functions in an integrin activation pathway initiated by IC binding to Fcγ receptors in addition to its better known role as a negative regulator of cell activation by G protein-coupled receptors. In contrast, LPL Ser5 phosphorylation and PMN adhesion induced by formylmethionyl-leucylphenylalanine or phorbol myristate acetate-induced LPL phosphorylation and activation of α5β2. Two phosphoinositidy 3-kinase inhibitors blocked FcγR-induced cAMP accumulation, demonstrating that this kinase acts upstream of PKA. These data demonstrate a necessary role for PKA in IC-induced integrin activation and LPL phosphorylation.

Leukocyte integrins are able to modulate avidity for their ligands. While circulating in blood or lymph, leukocytes maintain their integrins in a low adhesive state. In contrast, when leukocytes migrate out of the vasculature, their integrins have higher avidity, allowing them to participate in the processes of extravasation and migration through extracellular matrix. This regulation of integrin adhesion is essential for appropriate leukocyte function, since integrin activation is required for leukocyte migration to sites of inflammation and for lymphocyte recirculation through lymph nodes, but inappropriate activation leads to significant injury of normal tissues (1, 2). Many molecules specifically found at inflammatory sites or in lymph nodes can induce the transition of integrins from low to high avidity, and exposure to these molecules provides the stimulus both for transendothelial migration and for migration through the extracellular matrix to sites of infection and inflammation (3, 4).

The molecular mechanisms that modulate integrin avidity are not well understood. There is evidence for activation-induced increases in integrin affinity (5–7), integrin clustering (8), and integrin diffusion (9). At a molecular level, phosphoinositidy 3-kinase (PI 3-kinase)1 (10), protein kinase C (11, 12), and the Ca2+-dependent protease calpain (13) all can have a role in leukocyte integrin activation. In addition, there is evidence for involvement of the actin cytoskeleton in regulation of integrin avidity (9, 14). We recently demonstrated that cell-permeant peptides from the leukocyte-specific actin bundling protein L-plastin (LPL) will activate myeloid β2 integrins (15). These studies showed that LPL phosphorylation is closely associated with integrin activation and led to the hypothesis that LPL phosphorylation is a necessary step for integrin activation by many proinflammatory agents. This hypothesis has focused attention on the unknown enzyme(s) that phosphorylate LPL.

A well studied mechanism for leukocyte integrin activation is exposure to immune complexes (IC). IC binding to polymorphonuclear leukocyte (PMN) IgG Fc receptors (FcγR), especially FcγRIIA, induces integrin activation via a PI 3-kinase and PKC-dependent pathway, which is closely associated with LPL phosphorylation (12, 15). IC-induced activation of the leukocyte-specific integrin αmβ2 (Mac-1, CD11b/CD18) is required both for sustained adhesion to IC in vitro (12, 16) and for a normal IC-induced inflammatory response in vivo (17). Thus, IC-induced αmβ2 activation is probably a critical event in an

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‡ The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; FcγR, receptor for the Fc piece of IgG; fMLP, formylmethionyl-leucylphenylalanine; IC, immune complex(es); LPL, L-plastin; PKA, cAMP-dependent protein kinase A; PKC, cAMP-dependent protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil(s); mPMN, murine PMN; S5A, synthetic peptide based on amino acids 2–19 of LPL in which Ser5 has been changed to Ala; SCR, synthetic peptide in which amino acids 2–19 of LPL have been scrambled; PKC, protein kinase C; 8-Br-cAMP and 8-bromo-cyclic AMP and GMP, respectively; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin.
Jurkat cells (A) and PMN (B) are shown. A, Jurkat cells were treated with the indicated concentrations of 8-Br-cAMP. B, PMN were treated with buffer control (lanes 1 and 3), H89 (lanes 2 and 4), or wortmannin (lane 5) and then loaded to BSA- (lane 1) or IC- (lanes 3–5) coated surfaces or activated with PMA (lane 2) in suspension. LPL was immunoprecipitated as described under "Experimental Procedures." LPL phosphorylation was detected by γ-32P labeling (upper panel), or Western blotting with anti-phospho-LPL antibody (middle panel). Coomassie Blue staining of the two same gels showed equal loading of total immunoprecipitated LPL protein (lower panel).

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**Human PMN were isolated from the peripheral blood of healthy donors by dextran sedimentation and gradient centrifugation as described (18). Murine bone marrow PMN were purified by Percoll density gradient as described (19). Cells were resuspended in HBSS- + (1× Hank’s buffered salt solution with 20 mM Hepes, 8.9 mM sodium bicarbonate, 1.0 mM Mg2+, and 1.0 mM Ca2+) for all assays. A colony of CD18-deficient mice (a gift of Dr. Art Beaudet, Baylor University, Houston, TX) and congenic wild type or heterozygous mice were maintained as described previously (20). Jurkat T lymphoid cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mM l-glutamine, 0.1 mM nonessential amino acids, 50 mM 2-mercaptoethanol, and 100 μg/ml penicillin and streptomycin under a 5% CO2 atmosphere. F(ab’)2 fragments of IB4 (anti-CD18) were prepared as described (21). F(ab’)2 fragments of IV.3 (anti-CD32, anti-FcγRII) and 3G8 (anti-CD16, anti-FcγRIII) were purchased from Medarex, Inc. (Aannandale, NJ). Recombinant cGMP-dependent protein kinase (PKG), protein kinase C (PKC), casein kinase II, calmodulin-dependent kinase II, H89, KT5720, 8-Br-cAMP, 8-Br-cGMP, forskolin, KN-62, and DRB were from Calbiochem. Recombinant PKA was purchased from Sigma. Recombinant LPL protein was purified from bacterial lysates using immunoaffinity chromatography. Recombinant MK2 (MAPKAPK2), M3K (MAPKAPK3), and p38-regulated/activated protein kinase (p38 MAPK) plasmids were the kind gift of Dr. Jianhua Han (Scrpps Research Institute, La Jolla, CA). The sources of all other reagents have been described previously (12, 25).

LPL amino-terminal peptide and other mutant peptides were synthesized in the Protein Chemistry Laboratory (Washington University, St. Louis, MO). The following previously characterized peptides (15) were used in this study: amino acids 2–19 of human LPL (ARGAVSDEEMMELREAFA), a mutant peptide in which Ser⁵ has been changed to Ala (S5A) (ARGAVSDEEMMELREAFA), a mutant peptide in which Ser⁷ has been changed to Ala (S7A) (ARGSVDAEMMELREAFA), and a peptide in which the 19 amino acids have been scrambled (SCR) (AGDESEMVFMSALRE).

**Generation of Anti-phospho-LPL Antibody—**A polyclonal anti-phospho-LPL antibody was generated against a peptide encoding LPL amino acids 2–11 (ARGYSDEVEM) in which Ser⁵ was phosphorylated. The serum from immunized rabbits was collected and purified first by “negative” affinity on unphosphorylated peptide and then “positive” affinity on the phosphorylated peptide by Quality Controlled Biochemicals Inc. (Hopkinton, MA). Western blotting with the affinity-purified antiserum demonstrated that the antibody specifically recognized phosphorylated LPL from Jurkat cells as well as PMN but not the unphosphorylated protein and that the selectivity to phosphorylated LPL was similar to using autoradiography of LPL from 32P-loaded cells (Fig. 1).

This confirmed that in leukocytes Ser⁵ is a major LPL phosphorylation site, as previously demonstrated by transfection of HeLa cells (15).

**Adhesion Assay—**Tissue culture plates were coated with BSA, BSAanti-BSA IC, or fetal calf serum, and PMN were loaded with calcein as described previously (12). PMN were treated with control buffer or inhibitors for 30 min at 37 °C, following which 1×10⁶ cells/well were added to BSA- or IC-coated wells and incubated at 37 °C for the indicated times. For PMA- or FMLP-stimulated adhesion, PMA (50 ng/ml final concentration), FMLP (0.5 μM final concentration), or MeSO control was added after the addition of cells to BSA-coated wells. After the incubation period, the fluorescence (485-nm excitation and 530-nm emission wavelengths) was measured using a Max fluorescence plate reader (Molecular Devices, Sunnyvale, CA) before and after washing four times with 180 μl of phosphate-buffered saline. The percentage of adhesion was calculated by dividing the fluorescence after washing by the fluorescence before washing. In preliminary experiments, fluorescence was shown to be linearly related to cell number.

**In Vitro Phosphorylation Assay—**To assess peptide phosphorylation, purified kinases were mixed with 100 μM LPL, S5A, S7A, or SCR peptides in a reaction mixture containing 20 mM Tris, pH 7.4, 20 μM MgCl₂, 10 μM dithiothreitol, 100 μM ATP, and [γ-32P]ATP, incubated at 30 °C for 20 min. The reaction mixtures were spotted onto P81 phosphocellulose paper and washed four times in 75 mM phosphoric acid and once in 95% ethanol, following which retained radioactivity was determined. For in vitro LPL phosphorylation, 2.5 μg of purified recombinant LPL protein was mixed with purified kinases in the reaction mixture described above. After a 20-min incubation at 30 °C, the reaction was stopped by the addition of 100 °C SDS-polyacrylamide gel electrophoresis sample buffer, and protein components were resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and stained with Coomassie Blue to assess the protein loading. The membrane was then subjected to autoradiography to assess protein phosphorylation. Positive controls for the activity of each kinase (substrate peptides for PKA, PKG, PKC, and casein kinase II; myelin basic protein for Pak-1 and PKB; and autophosphorylation for M2K, M3K, and p38-regulated/activated protein kinase) were included in each assay to ensure that the kinase was active against known substrates.
Charles, MO) according to the manufacturer’s directions. The incubation procedure with the cAMP EIA kit (Linco Research Inc., St. Charles, MO) was incubated at 37 °C for 15 min at 4 °C. The supernatants were collected and dried in a vacuum oven. The dried extract material was dissolved in assay buffer, and the content of PKA was determined as described using the acetylation procedure with the cAMP EIA kit (Linco Research Inc., St. Charles, MO) according to the manufacturer’s directions.

RESULTS

cAMP-dependent Protein Kinase Specifically Phosphorylates L-plastin on Ser5 in Vitro—We have determined that Ser5 is the predominant LPL phosphorylation site (15). Since this serine is within a consensus sequence for phosphorylation by the cAMP-dependent protein kinase PKA (27), we tested whether PKA could phosphorylate LPL. We found that the purified catalytic domain of PKA phosphorylated an amino-terminal LPL peptide (containing LPL amino acids 2–19) but not the peptide in which Ser5 was mutated to Ala or a peptide in which LPL sequence 2–19 was scrambled (Fig. 2A). Mutation of Ser7 to Ala as a control did not block PKA phosphorylation (Fig. 2A). PKA also potently phosphorylated whole recombinant LPL protein in vitro (Fig. 2B). In contrast, PKG did not phosphorylate recombinant LPL (Fig. 2B) or LPL peptide (data not shown). While Ser5 also is within consensus sites for phosphorylation by PKC and casein kinase II, neither the catalytic domain of PKC nor casein kinase II phosphorylated recombinant LPL peptides or recombinant LPL protein in vitro (data not shown). In addition, we have tested Pak1, protein kinase B, and some downstream kinases in the mitogen-activated protein kinase pathway, such as M2K (MAPKAPK2), M3K (MAPKAPK3), and p38-regulated activated protein kinase (22–24); all failed to phosphorylate LPL or its amino-terminal peptide in vitro (Ref. 15 and data not shown). Thus, in contrast to other candidate kinases, PKA can specifically phosphorylate LPL on Ser5 in vitro.

PKA Activators Cause LPL Phosphorylation in Vivo—To examine whether PKA is involved in LPL phosphorylation in vivo, we tested the effects on LPL phosphorylation of the PKA activators 8-Br-cAMP, a cell-permeable cAMP analog (Fig. 3A), and forskolin, an adenylyl cyclase activator (Fig. 3C). Both induced LPL phosphorylation in a dose-dependent fashion in Jurkat cells. In contrast, the cell-permeant cGMP analog 8-Br-cGMP did not induce LPL phosphorylation even at 5 mM (data not shown). The increased LPL phosphorylation induced by 8-Br-cAMP or forskolin was readily inhibited by the PKA-specific inhibitor H89 (Fig. 3, B and C). 8-Br-cAMP also induced LPL phosphorylation in PMN, but only at a concentration of 5 mM (data not shown). Thus, PKA can directly phosphorylate LPL in vitro, and pharmacologic PKA activation can induce LPL phosphorylation in intact cells.

PKA Inhibitors Prevent IC Induction of LPL Phosphorylation—cAMP has most frequently been found to inhibit PMN activation (28–30), while LPL phosphorylation has been associated with PMN integrin activation (15). To investigate the potential significance of PKA in agonist-activated LPL phosphorylation, the effects of two PKA inhibitors, H89 (31) and KT5720 (32), were tested on LPL phosphorylation induced by IC, fMLP, or PMA. Both H89 and KT5720 potently inhibited LPL phosphorylation initiated by IC ligation of Fcγ receptors in PMN (Fig. 4, A and B). Neither the casein kinase II inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (33) nor the CaM kinase II inhibitor KN-62 (34) inhibited IC-induced LPL phosphorylation; demonstrating specificity of the effects of these two structurally unrelated PKA inhibitors. Moreover, neither PMA nor fMLP activation of LPL phosphorylation was inhibited by H89 or KT5720 (Fig. 4C and data not shown). These results suggest that PKA is responsible for IC-induced LPL phosphorylation in PMN but is probably not the kinase that phosphorylates LPL Ser5 in response to fMLP or PMA. PKA Is Required for Sustained PMN Adhesion to an IC-coated Surface, but Not for PMA- or fMLP-activated Adhesion—

FIG. 2. cAMP-dependent protein kinase specifically phosphorylates L-plastin on Ser5 in vitro. A, PKA catalytic domain phosphorylation of LPL peptide 2–19 (LPL), the same peptide in which Ser5 was replaced by Ala (SSA), the LPL peptide in which Ser5 was changed to Ala (STA), and a scrambled LPL peptide (SCR) was examined as described under “Experimental Procedures.” As negative controls, reaction mixtures were prepared without peptide substrate (no pep) or without kinase (no kinase). B, purified recombinant LPL was incubated with γ-32PATP and PKA or PKG as described under “Experimental Procedures.” LPL phosphorylation was analyzed by autoradiography (32P) as described in Fig. 1. Total loading of LPL was assessed by Coomassie Blue staining. The identity of the 70-kDa band in the PKG lanes is unknown, but it was in the recombinant PKG preparation.
Previous data suggest that LPL phosphorylation precedes αMβ2 activation, since IC, PMA, and fMLP all induce phosphorylation normally in the absence of expression of αMβ2 (25). Cell-permeant peptides containing the Ser5 phosphorylation site or constitutively phosphorylated Ser5 activate αMβ2, suggesting a role for LPL phosphorylation in integrin activation.

To assess the relevance of LPL phosphorylation by PKA for integrin activation, we investigated the effects of PKA inhibitors on αMβ2-mediated adhesion in PMN. While initial adhesion of PMN to an IC-coated surface requires only Fcy receptors (12, 16), H89 inhibited IC-induced adhesion significantly at a late time point (30 min) but had no effect on early PMN attachment (Fig. 5A), which is identical to the defect of β2-deficient PMN (16). The kinetics of attachment to IC by H89-treated PMN parallel those of wortmannin-treated PMN (Fig. 5A). H89 inhibited IC-induced LPL phosphorylation (Fig. 5, inset, Fig. 4, and Ref. 15). Furthermore, the dose response of H89 inhibition of LPL phosphorylation correlated closely to the inhibition of sustained IC-induced adhesion (Fig. 5B), consistent with the hypothesis that PKA phosphorylation of LPL is a required step in IC-induced activation of αMβ2. In contrast, neither H89 nor KT5720 inhibited fMLP or PMA activation of PMN αMβ2-mediated adhesion (Fig. 5, C and D). In fact, H89 or KT5720 moderately increased both control and fMLP-induced adhesion to BSA or fetal calf serum-coated surfaces (Fig. 5, C and D, and data not shown), in agreement with previous studies demonstrating that cAMP inhibits fMLP-induced PMN activation.

**FIG. 3.** PKA activators cause LPL phosphorylation, which is inhibited by the PKA-specific inhibitor H89. A, Jurkat cells were loaded with [32P]phosphoric acid prior to treatment with the indicated concentration of 8-Br-cAMP for 30 min at 37 °C. LPL phosphorylation was assessed by autoradiography; LPL loading was assessed by Coomassie Blue staining. B, Jurkat cells were treated with various concentrations of H89 as indicated for 30 min at 37 °C and then incubated with 1 mM 8-Br-cAMP or buffer for an additional 30 min. LPL phosphorylation was detected by Western blotting with the anti-phospho-LPL antibody as described in Fig. 1. LPL loading was assessed by Coomassie Blue staining. C, same as B, except that cells were treated with the indicated concentrations of forskolin.

**FIG. 4.** H89 inhibits IC-, but not PMA- or fMLP-activated LPL phosphorylation. A, purified human PMN were loaded with [32P]phosphoric acid, treated for 30 min with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; 100 μM), H89 (50 μM), KN-62 (100 μM), or DMSO (DMSO) as control prior to incubation on BSA- or IC-coated surfaces as described under “Experimental Procedures.” LPL phosphorylation was assessed by autoradiography. B, PMN were treated with KT5720 (5 μM) or DMSO prior to incubation on BSA- or IC-coated surfaces. LPL phosphorylation was assessed by immunoblotting with anti-phospho-LPL Ab. The loading of total LPL was assessed by immunoblotting the same membrane with LPL4A.1. C, PMN were treated with H89 (50 μM) or control and then left unstimulated or stimulated with fMLP (500 nM) or PMA (50 ng/ml) for 10 min. LPL phosphorylation was assessed by Western blot.
FIG. 5. PKA inhibitors block PMN adhesion to IC-coated surfaces but have no effect on PMA- or fMLP-activated adhesion. A, purified human PMN were treated with H89 (50 μM), wortmannin (100 nM), or buffer prior to incubation on BSA- or IC-coated surfaces for the indicated times at 37 °C. ○, BSA; ■, IC; ▲, IC plus H89; ●, IC plus wortmannin. The data are the mean ± S.E. of triplicate wells, reported as the percentage of adherent cells after washing. Inset, a portion of cells at the 30-min time point were taken, and LPL phosphorylation was assessed as described under “Experimental Procedures.” B, left, PMN were treated with varying concentrations of H89 and allowed to adhere for 30 min to BSA- or IC-coated surfaces, and LPL phosphorylation was measured. Right, PMN were treated identically to those in the left panel, and adhesion assays were performed as described under “Experimental Procedures.” The dotted line represents the quantitation of the density of each band in the left panel (except the BSA band, which was not included). ▲, BS; ○, IC; ○, LPL phosphorylation. C, PMN, preincubated with H89 (open bars; 50 μM) or buffer (filled bars), were incubated with BSA-coated surfaces with PMA (50 ng/ml), fMLP (500 nM), or buffer. Adhesion was quantitated as above. D, same as C except that cells were treated with KT5720 (filled bars; 5 μM) or Me₂SO (DMSO; open bars) control. The data in each panel are representative of at least three independent experiments.
PKA in Neutrophil Integrin Activation

PKA Is Required for Fc Receptor-induced α5β2 Integrin Activation—The time course of H89 inhibition of IC-induced adhesion (Fig. 5A) showed that H89 did not inhibit early adhesion but completely inhibited adhesion after 30 min, indicating that PKA probably has no role in FcγR-mediated initial adhesion but is involved in activation of α5β2 by FcγR ligation. To further test this hypothesis, we examined the effects of PKA inhibitor H89 on IC-induced adhesion in PMN from mice with β2 integrin deficiency due to gene disruption by a neomycin cassette (20). β2 integrin-deficient PMN can only adhere to IC for a short period of time and then fall off because α5β2 is required for sustained adhesion to IC (16, 17). In murine PMN (mPMN), even early FcγR-mediated adhesion is dependent on α5β2. Therefore, β2 integrin-deficient mPMN adhere to IC less well than wild type even in early time points. Nonetheless, β2 integrin-deficient mPMN adhered specifically to IC. Adhesion was transient and disappeared after 30–40 min (Fig. 6A). H89 had no effect on adhesion of these mPMN to IC but inhibited sustained adhesion in wild type mPMN, as it had in human PMN (Fig. 6, A and B). Adhesion of H89-treated wild type mPMN was reduced to the level of β2 integrin-deficient mPMN (Fig. 6B). These results prove that PKA is not required for initial FcγR-mediated adhesion but is necessary for FcγR-induced α5β2 integrin activation.

Ligation of Fcγ Receptors Transiently Increases Intracellular cAMP Level in PMN—To determine whether IC-induced activation of PKA resulted from an increase in intracytoplasmic cAMP concentration ([cAMP]), cytoplasmic [cAMP] was measured at various times after IC activation. [cAMP] was transiently increased when PMN adhered to IC, with a peak at 5 min, and then returned to its initial level after 15 min (Fig. 7A). The increase in cAMP was inhibited by anti-FcγRI antibody IV.3, confirming its initiation by FcγR ligation. As expected, PKA inhibitor H89 or anti-β2 antibody IB4 treatment did not inhibit this increased cAMP level (Fig. 7B), demonstrating that cAMP elevation requires FcγRIIA ligation but precedes PKA and integrin activation.

Since PI 3-kinase inhibitors blocked sustained adhesion to IC with similar kinetics as PKA inhibitors and also blocked LPL phosphorylation (Fig. 5A andRefs. 12 and 15), it was important to determine whether PI 3-kinase is necessary for PKA activation by IC. Both wortmannin and LY294002, two PI 3-kinase inhibitors with different modes of action, blocked IC-induced increase in cAMP (Fig. 7A), suggesting that PI 3-kinase functions upstream of PKA in the pathway for LPL phosphorylation and integrin activation, perhaps in regulation of adenylate cyclase.

Fig. 6. Adhesion of murine PMN to IC. A, bone marrow PMN from β2 integrin-deficient mice (β2−/− mPMN) were allowed to adhere to BSA- or IC-coated surfaces in the presence of increasing concentrations of H89. Cell adhesion was quantitated as described under “Experimental Procedures.” ◊, BSA; □, IC; △, IC plus H89 (25 μM); ○, IC plus H89 (50 μM). B, adhesion to IC of β2−/− and wild type mPMN was compared in the absence and presence of H89. Cell adhesion was quantitated as described above. The data are from one of three independent experiments with similar results. ▲, wild type plus buffer; ■, wild type plus H89 (25 μM); △, β2−/− plus buffer; □, β2−/− plus H89 (25 μM); ○, wild type plus H89 (50 μM).

Fig. 7. IC adhesion transiently increases intracellular cAMP in PMN. A, PMN were treated with wortmannin (100 μM) or LY294002 (25 μM) or Me2SO control for 15 min and then allowed to adhere to surfaces coated with BSA or IC for the indicated times. [cAMP] was determined as described under “Experimental Procedures.” Each point represents the average of duplicates. ◊, BSA; ○, IC; △, IC plus wortmannin; □, IC plus LY294002. B, PMN were pretreated with F(ab)′/IgIV.3 anti-FcγRII (20 μg/ml), F(ab)′/IgV anti-β2 (20 μg/ml), H89 (50 μM), or buffer and allowed to adhere to BSA- or IC-coated surfaces. [cAMP] in each sample was determined after 5 min. The data are representative of three independent experiments.
**PKA in Neutrophil Integrin Activation**

**DISCUSSION**

Previous work using cell-permeant peptides from the amino terminus of LPL showed that these peptides were capable of inducing rapid integrin activation in PMN (15). Integrin activation by these peptides required Ser5 and was prevented by PI 3-kinase inhibitors. Surprisingly, integrin activation by a cell-permeant peptide in which Ser5 was phosphorylated was independent of PI 3-kinase, leading to the hypothesis that the well described role for PI 3-kinase in integrin activation (10) results from its involvement in a pathway resulting in phosphorylation of Ser5 of LPL. These studies focused attention on the phosphorylation of LPL Ser5 as a potentially critical regulatory step in the activation process (44) and a component of the regulation of phagocytosis (45). Nonetheless, LPL phosphorylation by high concentration 8-bromocAMP was shown to block FcγRIIA-induced, LPL phosphorylation, and integrin activation (12, 15). Our data demonstrate that PI 3-kinase inhibitors block FcγR-induced cAMP accumulation, which is consistent with the hypothesis that the role for PI 3-kinase is upstream of PKA activation in IC-induced integrin activation. While this could theoretically be either through regulation of adenylate cyclase or phosphodiesterase activity, the PI 3-kinase inhibitors blocked cAMP accumulation when phosphodiesterase activity was inhibited by isobutylmethylxanthine, strongly implicating PI 3-kinase in activation of adenylate cyclase. While the signaling pathway responsible for this regulation is unknown, there is evidence that the state of assembly of the actin cytoskeleton, which can be regulated by PI 3-kinase, affects adenylate cyclase activity in S49 cells (49) and in yeast (50, 51). It is intriguing as well that an adenylate cyclase regulatory protein in Dictostelium contains a pleckstrin homology domain (52), since these domains often bind inositol 1,4,5-trisphosphate, the product of PI 3-kinase activity. Whatever the precise mechanism, these studies have identified adenylate cyclase as a physiologically significant downstream target of PI 3-kinase after its activation by FcγR ligation.

The hypothesis that LPL phosphorylation is a requisite step in PMN integrin activation suggests that interference with its phosphorylation should block activation-dependent integrin-mediated adhesion. Since PKA inhibitors blocked LPL phosphorylation by IC, we tested the effects of these inhibitors on αβ2 integrin activation by examining sustained adhesion to IC. These inhibitors blocked IC-induced, αβ2-dependent sustained adhesion of both human and murine PMN. The specificity of the effect was demonstrated by (i) failure of inhibitors of other serine kinases to affect αβ2 activation; (ii) failure of the PKA inhibitors to block the transient FcγR-mediated adhesion to IC that occurs in αβ2-deficient PMN; and (iii) failure of the PKA inhibitors to block the αβ2-mediated adhesion induced by FMLP and PMA, which cause PKA-independent LPL phosphorylation. Thus, these data are further evidence for a close association between LPL phosphorylation and PMN integrin activation and support the hypothesis that LPL phosphorylation (which occurs normally in αβ2-deficient cells (25)) is a required step in activation of αβ2-mediated adhesion.

A role for cAMP in phagocyte activation is quite unexpected. In general, increases in cAMP have been found to inhibit FMLP-stimulated PMN activation, as measured by adhesion or respiratory burst activation (30, 36, 40, 41) and have been found to inhibit integrin activation in several cell types (29, 42, 43). However, FcγR-mediated accumulation of cAMP and of PKA at phagosomes has been noted previously (44, 45), although this cAMP has been variously interpreted as part of the activation process (44) and a component of the regulation of phosphocytosis (45). A resolution of the apparent conflict between the well described negative regulatory role for cAMP in FMLP stimulation and its activating role in FcγR-mediated signaling may lie in the compartmentalization of cAMP in response to FcγR ligation. Localized increases in cAMP may be prevented from propagating through the cytoplasm by the colocalization of phosphodiesterase (45), and it is of note that in the present study increases in cytosolic cAMP on adhesion to IC could only be detected in the presence of a phosphodiesterase inhibitor. Pharmacologic increases in cAMP or increases that occur because of ligation of receptors (28) at a distance from the adhesion site may activate quite different processes than cAMP confined to the region of FcγR ligation, because of access to different downstream signaling pathways. In this regard, it is potentially interesting that LPL is phosphorylated by 8-bromocAMP addition in PMN only at very high concentrations, consistent with the possibility of divergent effects of this mediator on the different pathways leading to LPL phosphorylation.

Finally, our data suggest that PI 3-kinase is involved in an FcγR-initiated pathway to PKA activation, since wortmannin and LY294002 both block PKA-mediated LPL phosphorylation in PMN and FcγR-induced increase in cAMP. Activation of PI 3-kinase by PMN FcγR ligation is well described (46–48), and inhibition of PI 3-kinase has been shown to block FcγR-mediated phagocytosis (47), LPL phosphorylation, and integrin activation (12, 15). Our data demonstrate that PI 3-kinase inhibitors block FcγR-induced cAMP accumulation, which is consistent with the hypothesis that the role for PI 3-kinase is upstream of PKA activation in IC-induced integrin activation. While this could theoretically be either through regulation of adenylate cyclase or phosphodiesterase activity, the PI 3-kinase inhibitors blocked cAMP accumulation when phosphodiesterase activity was inhibited by isobutylmethylxanthine, strongly implicating PI 3-kinase in activation of adenylate cyclase. While the signaling pathway responsible for this regulation is unknown, there is evidence that the state of assembly of the actin cytoskeleton, which can be regulated by PI 3-kinase, affects adenylate cyclase activity in S49 cells (49) and in yeast (50, 51). It is intriguing as well that an adenylate cyclase regulatory protein in Dictostelium contains a pleckstrin homology domain (52), since these domains often bind inositol 1,4,5-trisphosphate, the product of PI 3-kinase activity. Whatever the precise mechanism, these studies have identified adenylate cyclase as a physiologically significant downstream target of PI 3-kinase after its activation by FcγR ligation.

In summary, this work has two significant implications for understanding the biochemical pathways that lead to PMN integrin activation. First, these studies identify a PKA-dependent activation pathway initiated by IC. Although PKA has generally been thought to negatively regulate PMN activation, most of these studies have been done primarily with FMLP-induced activation, and the current data are completely compatible with the previously observed divergence between IC- and FMLP-induced PMN activation (12). Furthermore, IC-initiated PKA activation is dependent on PI 3-kinase via its regulation of adenylate cyclase, a novel pathway for regulation of cytosolic cAMP.

Second, these data support the hypothesis that LPL phosphorylation is a requisite step in PMN integrin activation. PKA inhibitors block IC-induced integrin activation but not FMLP- or PMA-induced activation, which is in perfect accord with their effects on LPL phosphorylation. Since PKA was identified as a candidate for involvement in integrin activation because of its ability to phosphorylate LPL in *vitro*, these data strongly support the involvement of LPL phosphorylation in integrin phosphorylation by the PKC catalytic domain as a potentially critical regulatory step in activation of PMN integrins. In the present work, we present data indicating that PKA can phosphorylate LPL and does so when PMN FcγRIIA is ligated. First, PKA phosphorylates both LPL and an LPL amino-terminal peptide in *vitro*. PKA phosphorylation occurs on Ser5, as determined by failure of phosphorylation of the S5A mutant peptide and by recognition of PKA-phosphorylated LPL by a phospho-Ser5-specific antibody. Second, agents that increase intracytoplasmic cAMP, which activates PKA, induce LPL phosphorylation. Finally, two pharmacologic PKA inhibitors block IC-induced LPL phosphorylation in PMN. While the data in whole cells demonstrate only that PKA activation can lead to LPL phosphorylation, together with the demonstration of direct phosphorylation in *vitro*, these data support the hypothesis that PKA can directly phosphorylate LPL in cells. It is likely that other kinase(s) can phosphorylate LPL as well, since FMLP and PMA-induced phosphorylation are not antagonized by the same PKA inhibitors that block IC-induced phosphorylation. While these kinases are not known, PKC is not a likely candidate, since neither recombinant LPL nor the LPL amino-terminal peptide was phosphorylated by the PKC catalytic domain in *vitro*. The existence of distinct kinases that can phosphorylate LPL is consistent with previous data suggesting two distinct signaling pathways for both LPL phosphorylation and integrin activation (12, 15).

In summary, this work has two significant implications for understanding the biochemical pathways that lead to PMN integrin activation. First, these studies identify a PKA-dependent activation pathway initiated by IC. Although PKA has generally been thought to negatively regulate PMN activation, most of these studies have been done primarily with FMLP-induced activation, and the current data are completely compatible with the previously observed divergence between IC- and FMLP-induced PMN activation (12). Furthermore, IC-initiated PKA activation is dependent on PI 3-kinase via its regulation of adenylate cyclase, a novel pathway for regulation of cytosolic cAMP.

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activation. While additional methods of approach will be required to define the mechanism by which LPL phosphorylation affects integrin function, it is highly likely that this is a significant regulatory step in the regulation of leukocyte integrin avidity.

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