β_2 Integrin Engagement Triggers Actin Polymerization and Phosphatidylinositol Trisphosphate Formation in Non-adherent Human Neutrophils

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Abstract. β_2 integrins are involved in the adhesion of leukocytes to other cells and surfaces. Although adhesion is required for cell locomotion, little is known regarding the way β_2 integrin-receptors affect the actin network in leukocytes. In the present study filamentous actin (F-actin) levels in non-adherent human neutrophils have been measured by phalloidin staining after antibody cross-linking of β_2 integrins. Antibody engagement of β_2 integrins resulted in a rapid and sustained (146 and 131% after 30 and 300 s, respectively) increase in the neutrophil F-actin content. This is in contrast to stimulation with N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP), which causes a prompt and pronounced but rapidly declining rise in F-actin (214 and 127% after 15 and 300 s, respectively). Priming neutrophils with 1 nM PMA, a low concentration that did not influence the F-actin content per se, increased the magnitude of the β_2 integrin-induced response but had no effect on the kinetics (199% after 30 s and 169% after 300 s). Removal of extracellular Ca^{2+} only marginally affected the β_2 integrin-induced F-actin response for cells that were pretreated with PMA whereas the response for nonprimed cells was reduced by half. This suggests that even though extracellular Ca^{2+} has a modulatory effect it is not an absolute requirement for β_2 integrin-induced actin polymerization. β_2 integrin engagement did not affect the resting cellular level of cAMP arguing against a role of cAMP in β_2 integrin-induced actin assembly. The lack of a cAMP signal might instead explain, at least in part, the prolonged F-actin response triggered by β_2 integrins, since addition of cAMP and 1-isobutyl-methylxanthine (IBMX) caused a prompt reversal of the β_2 integrin-induced F-actin elevation in electropermeabilized neutrophils. Engagement of β_2 integrins, as previously shown for activation of the chemotactic peptide receptor, resulted in a significant formation of PtdInsP$_3$. The capacity of β_2 integrins and chemotactic peptide receptors to induce phosphatidylinositol trisphosphate (PtdInsP$_3$) formation correlated with their ability to induce actin polymerization.

THE ability to destroy invading microorganisms during infection is critically dependent on the chemotactic and phagocytic properties of neutrophils. These motile processes are driven by a continuous remodeling of the microfilamentous system (Stossel, 1992). Actin, the major component of this system can rapidly interconvert between globular (G-actin)[1] and filamentous (F-actin) forms (Korn, 1982). In response to a variety of stimuli, such as the chemotactic peptide N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP), an increased assembly of actin filaments takes place in the neutrophil cytosol (Wallace et al., 1984; for review see Omann et al., 1987). So far, the signal transduction mechanisms that regulate actin assembly and disassembly in neutrophils during locomotion have been only partly elucidated.

Second messenger generation induced by fMLP occurs via a pertussis–toxin-sensitive heterotrimeric GTP-binding protein (G-protein). Pertussis toxin-mediated inactivation of these G-proteins inhibits chemoattractant-triggered actin polymerization (Becker et al., 1985; Bengtsson et al., 1986), and direct activation of G-proteins by GTPγS or AlF$_4^-$ in permeabilized neutrophils results in increased actin assembly (Therrien and Naccache, 1989; Downey et al., 1989; Bengtsson et al., 1990). Both of these findings clearly show the importance of G-protein(s) in chemotactic factor-mediated actin assembly. The idea of a close association between G-proteins and cytoskeletal modulation was recently extended in studies of fibroblasts, where members of the *rho* subfamily of small G-proteins were shown to play a crucial role in growth factor-induced membrane ruffling and stress

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1. *Abbreviations used in this paper:* F-actin, filamentous actin; fMLP, N-formyl-l-methionyl-l-leucyl-l-phenylalanine; G-actin, globular actin; G-protein, GTP-binding protein; IBMX, 1-isobutyl-methylxanthine; PtdIns(4,5)P$_2$, phosphatidylinositol (4,5)bisphosphate; PtdInsP$_3$, phosphatidylinositol trisphosphate; RAM, rabbit anti-mouse immunoglobulins.

fiber formation (Ridley et al., 1992; Ridley and Hall, 1992). Less understood, however, are the signaling events/effector systems that follow agonist-induced activation of G-protein(s) and are responsible for triggering actin polymerization (Downward, 1992; Goldschmidt-Clermont et al., 1992; Stossel, 1992).

Resting leukocytes contain a large pool of G-actin (Fechheimer and Zigmond, 1983) which is inhibited from spontaneous polymerization by capping proteins such as gelsolin (Stossel, 1992) and by binding to actin-binding proteins such as profilin, thymosin β4 and β10 (Lassing and Lindberg, 1985; Cassimeris et al., 1992; Yu et al., 1993; for review see Stossel, 1992). Control of actin polymerization in response to signals generated by cell stimulation is often considered to occur through uncapping of the barbed ends of F-actin and/or release of G-actin from sequestering proteins (Stossel, 1992). It has been demonstrated in cell-free systems that phosphatidylinositol(4,5)bisphosphate (PtdIns-(4,5)P₂) dissociates profilactin complexes and inactivates the capping and severing function of gelsolin and thereby accelerating actin polymerization (Lassing and Lindberg, 1985, 1988; Janmey et al., 1987; Janmey and Stossel, 1987). These findings and observations performed on thrombin-stimulated platelets suggest that an increased turnover, or net increase of PtdIns(4,5)P₂ can regulate actin assembly (Lassing and Lindberg, 1990). In addition, profilin may also play an important role in cell signaling since the interaction between PtdIns(4,5)P₂ and profilin has been shown to inhibit profilin's interaction with actin as well as the hydrolysis of PtdIns(4,5)P₂ by soluble PLCs (Goldschmidt-Clermont et al., 1990) but not tyrosine phosphorylated PLCγ-1 (Goldschmidt-Clermont et al., 1991).

Although agonist-induced turnover of PtdIns(4,5)P₂ is regulated via G-protein activation by many receptors, an increased interest has recently been focused on another phosphoinositide, phosphatidylinositol trisphosphate (PtdInsP₃) in relation to the regulation of the actin network. This lipid is generated from PtdIns(4,5)P₂ via activation of a PtdIns 3-kinase (Stephens et al., 1991), a heterodimeric enzyme comprised of 110- and 85-kD subunits (Otsu et al., 1991; Escobedo et al., 1991; Skolnik et al., 1991; Hiles et al., 1992). The activation of this enzyme has been extensively studied in relation to growth factor signal transduction and cell transformation associated with an increased tyrosine phosphorylation (Auger and Cantley, 1991; Cantley et al., 1991). However, activation of the chemotactic peptide receptor on neutrophils causes activation of the PtdIns 3-kinase via a pertussis sensitive G-protein (Traynor-Kaplan et al., 1989), by a mechanism that does not seem to require tyrosine phosphorylation (Vlahos et al., 1992).

Neutrophils can move and spread on a surface even in the absence of a chemoattractant gradient, although this movement is not directed. Interestingly, Southwick and co-workers (1989) observed that actin polymerization induced by adhesion to a plastic surface requires the presence of extracellular Ca²⁺ but was not mediated through a pertussis toxin sensitive G-protein and thereby differs from polymerization elicited by chemoattractants. In addition, the actin reorganization observed in adhering neutrophils has been proposed to correlate with a decrease in the cellular content of cAMP (Nathan and Sanchez, 1990). Although antibodies to adhesion receptors can block this decrease, neutrophils lacking adhesion receptors are still able to lower their cAMP level upon adhesion to a surface (Nathan and Sanchez, 1990). Consequently, the role of adhesion receptors in the observed cAMP response in adhering neutrophils remains unclear.

The CD11b/CD18 integrin, which belongs to the β₂ integrin family, is the numerically and functionally dominant adhesion receptor of human neutrophils (Hynes, 1987). The repeated elevations of cytosolic free calcium originally observed by Jaconi et al. (1988), are considered to be mediated by CD11b/CD18 integrins (Richter et al., 1990a; Jaconi et al., 1991). The transmembrane signaling capacity of the CD11b/CD18 integrin is further supported by the finding that specific engagement of this receptor by antibody cross-linking, to mimic a state of adhesion in suspended cells, induces a prompt calcium response (Ng-Sikorski et al., 1991).

The aim of the present investigation was to specifically engage β₂ integrins on non-adherent neutrophils by antibody cross-linking in order to study the effect of these adhesion molecules on actin polymerization.

Materials and Methods

Materials

All reagents used were of analytical grade. Dextran and Ficoll-paque were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Butylated hydroxytoluene (BHT), 1-isobutyl-methylxanthine (IBMX), cAMP, fMLP, phosphoinositides, lysophosphatidylcholine, and anti-BSA were from Sigma Chemical Co. (St. Louis, MO). The cAMP-kit and ³²P (PBS-13) were obtained from Amersham (Amersham International, Amersham, Bucks., UK). Pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA). Phorbol 12-myristate 13-acetate (PMA) and 4-α-PMA were from LC Serv. Corp. (Woburn, MA). Fluorescein phalloidin and fura2 free acid were obtained from Molecular Probes Inc. (Eugene, OR). The mAb IB4 anti-CD18 (IgG₂ₐ) originated from Dr. S. Wright (Rockefeller University, New York) (Wright et al., 1983) and the rabbit anti-mouse immunoglobulins (RAM) from Dakopatts (Glostrup, Denmark).

Isolation of Neutrophils

Blood from healthy volunteers was collected in heparin-containing tubes, and the neutrophils were isolated essentially as previously described (Böyum, 1968). In short, after elimination of erythrocytes by dextran sedimentation followed by a brief hypotonic lysis, the cell suspension was centrifuged on a Ficoll Paque gradient to separate the polymorphonuclear leukocytes from lymphocytes, monocytes, and platelets. The polymorphonuclear leukocytes were then washed twice in a calcium-free Krebs-Ringers glucose solution before being resuspended in a calcium-containing medium: 136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 0.1 mM EGTA, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 5.5 mM glucose, and 20 mM Hepes, pH 7.4. In some experiments, CaCl₂ was excluded from this medium. These two media are referred to as calcium-containing medium and calcium-free medium. This isolation procedure yielded a preparation that contained >95% neutrophils as determined from stained smears.

Electropermeabilization of Neutrophils

Cells were suspended (1 × 10⁷ cells/ml) in a medium with the following composition: 120 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 10 mM Pipes, 3 mM EGTA, and CaCl₂. The free Ca²⁺ concentration was buffered with EGTA to a theoretical concentration of 100 nM by mixing equimolar solutions of EGTA and Ca²⁺-EGTA in proportions calculated by using a computer program (LIGAND; Tatham and Gomperts, 1990). The concentration of free Ca²⁺ was then checked, and if necessary corrected, in a spectrofluorometer by using fura2 free acid (Fällman et al., 1992). Aliquots (1 ml) of the cell suspension were exposed to 25–30 electrical discharges (150 μs each) of 1.7 kV/cm (Särndahl et al., 1989; Fällman et al., 1992). The cells were kept on ice during the entire permeabilization procedure. The de-

gree of cellular permeabilization was estimated by staining with trypan blue (Särndahl et al., 1989; Fällman et al., 1992). In the present study 20-40% of the cells were stained after 25-30 discharges.

Activation of β_2 Integrins by Antibody Cross-linking

Human neutrophils (6 × 10^6 cells/ml) were incubated with IB4 (10 μg/ml), a mAb against CD18, for 20 min at 37°C in calcium-containing or, where indicated, calcium-free medium. The cells were then pelleted and resuspended in prewarmed volumes of the specified media. Cross-linking of the IB4 antibody was then obtained by the addition of RAM (1:50 dilution). Control experiments were performed using anti-BSA or F(ab')₂ fragments of RAM to exclude that the observed responses were a result of Fc-receptor engagement. The anti-BSA tested in these experiments was chosen because it is, when aggregated, very effective in activating Fc-receptors on suspended neutrophils (Rosales and Brown, 1992). The control results revealed that adding anti-BSA to suspended neutrophils preincubated with the IB4 antibody did not cause any of the effects seen upon addition of intact RAM whereas addition of F(ab')₂ fragments of RAM did (data not shown).

Determination of the Cellular Content of F-actin

The cellular content of F-actin was analyzed by staining with fluorescein phalloidin, essentially as previously described (Wallace et al., 1984; Bengtsson et al., 1986, 1988). Suspended cells were either stimulated with fMLP (100 nM) or subjected to cross-linking of β_2 integrins for different periods of time. Aliquots were then fixed with 4% (wt/vol) paraformaldehyde on ice, and then stained in the dark at room temperature for 30 min by incubation in a mixture of lysophosphatidylcholine (200 μg/ml) and fluorescein phalloidin (0.6 μg/ml). After washing and resuspension in PBS the fluorescence of the different samples was analyzed with a spectrofluorometer.

Determination of the Cellular Content of cAMP

Immediately before and after either stimulation with fMLP (100 nM) or cross-linking of β_2 integrins for different periods of time, aliquots (100 μl) of the respective cell suspension were transferred to ice-cold ethanol (final concentration 65%, vol/vol) to stop the reaction. Each sample was then allowed to settle for 1 h at −20°C, and the supernatant, which contained cAMP, was subsequently collected, as previously described (Fällman et al., 1989). The cAMP content was then determined by using a commercial assay kit (Amersham International).

Incubation with Pertussis Toxin

Neutrophils (6 × 10^6/ml) were incubated for 90 min at 37°C with 400-1,000 ng/ml pertussis toxin. Incubation with the monoclonal IB4 antibody (20 min at 37°C) and PMA (1 nM, at 37°C, for the last 10 min) for cross-linking experiments was carried out immediately after the incubation with pertussis toxin.

Determination of the Cellular Contents of ^{32}P-PtdInsP₃

Neutrophils (5 × 10^7/ml) were incubated for 90 min at 37°C with ^{32}P (2-3 mCi/ml) in calcium- and phosphate-free medium with supplements, as described in Results and in the legend of Fig. 6. The cells were then pelleted and resuspended in prewarmed calcium-containing medium and either stimulated with fMLP (100 nM) or subjected to activation of the β_2 integrins. The stimulations were terminated after the periods of time indicated in the legend of Fig. 6 by adding 500 μl of the cell suspension to 3 ml of chloroform:methanol (1:2, vol/vol) containing the antioxidant BHT (0.63 mg/ml) and phosphoinositides (10 μg/ml) as cold carrier. The phospholipids were then extracted as previously described (Traynor-Kaplan et al., 1989) and deacylated with methyl amine reagents to obtain glycerophosphoinositol phosphates (Clarke and Dawson, 1981). The different glycerophosphoinositol phosphates were subsequently separated by HPLC using a Partisil 10 SAX column (Phenomenex, Torrance, CA) and an NH₄HCO₂, pH 3.7, gradient (Nolan and Lapetina, 1990; Sjölander et al., 1991). Radioactivity in the elutes was monitored with an on-line radioactivity flow detector Series A-200 (Radiomatic Instruments, Tampa, FL). Standards were obtained by deacylation of ^3H-labeled PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ to yield their corresponding glycerophosphoinositol phosphates.

Results

### β_2 Integrin Engagement Induces Actin Polymerization in Non-adherent Neutrophils

To evaluate the role of β_2 integrins in adhesion induced actin polymerization, we specifically activated these receptors in non-adherent neutrophils by antibody cross-linking. This activation caused an increase in the cellular F-actin content that peaked after 30 s at 146% of basal (Fig. 1). In comparison, an fMLP-induced F-actin response was faster and more pronounced, peaking after 15 s at 214% (Fig. 1). Furthermore, the decline of the β_2 integrin-induced response differed from that of the fMLP-induced response: after 5 min, the F-actin content was still 67% of peak value considering the former but only 24% for the latter (Fig. 1). Control experiments showed that addition of either the IB4 antibody (anti-CD18) or RAM alone did not affect the cellular F-actin content (Fig. 1).

Effects of Pre-exposure to PMA or 4-α-PMA on β_2 Integrin-induced Actin Polymerization

Since the addition of PMA is known to enhance C3bi-mediated phagocytosis via the CD11b/CD18 receptor (Wright and Meyer, 1986), we investigated whether PMA could potentiate the β_2 integrin-induced effect on the cellular content of F-actin. Such potentiation did occur in β_2 integrin-
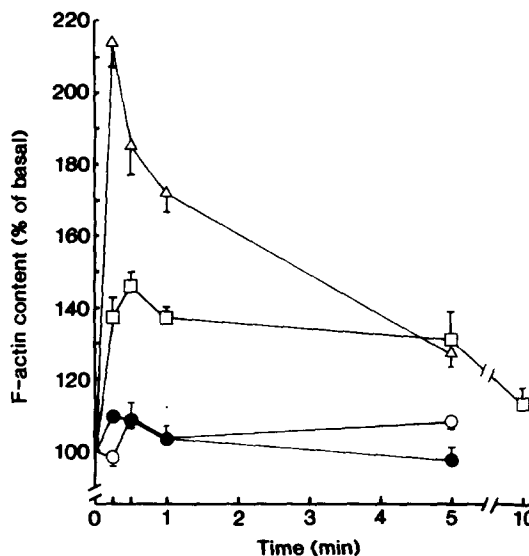
Figure 1. Effects of β_2 integrin engagement and stimulation with fMLP on the cellular content of F-actin. Human neutrophils (6 × 10^6 cells/ml) were incubated for 5 min at 37°C in calcium-containing medium and subsequently exposed to fMLP (100 nM, n = 10-12, △). Engagement of β_2 integrins was effected by first incubating the cells with the IB4 antibody (anti-CD18, IgG₂ₐ, 10 μg/ml) for 20 min at 37°C and then, after washing, adding RAM (1:50 dilution, n = 8-9, □). Control experiments were performed by adding the IB4 antibody (10 μg/ml, n = 2-3, ○) or RAM (1:50 dilution, n = 2-3, ●) alone. Error bars not indicated fall within the range of the symbols. Samples were taken at the indicated times and the cellular F-actin content was determined as described in Materials and Methods. Data are given as mean ± SEM and expressed as percent of the time-zero value for each individual experiment.
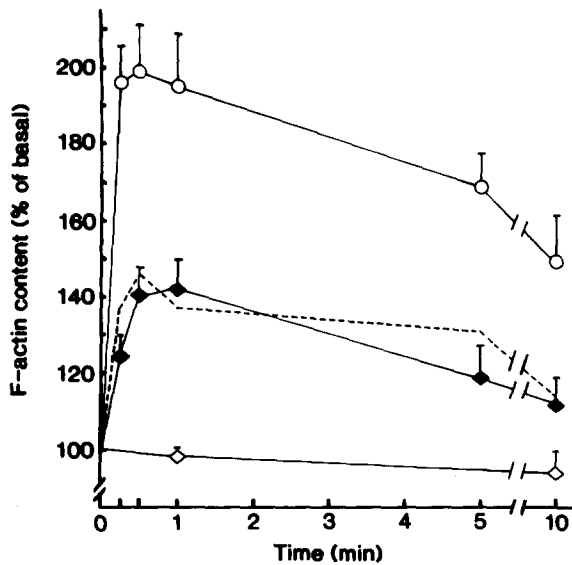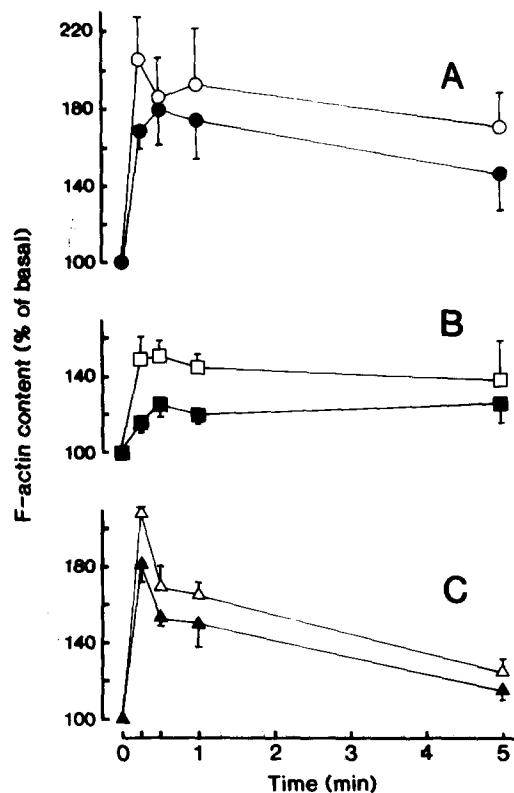
Figure 2. Effects of PMA and 4-α-PMA on β_2 integrin-induced actin polymerization. Human neutrophils (6 × 10⁶ cells/ml) were incubated with PMA (1 nM, n = 8-9, ○) or 4-α-PMA (1 nM, n = 3, ◆) for 10 min at 37°C. The cells were then washed and resuspended in prewarmed calcium-containing medium and incubated with the IB4 antibody (anti-CD18, IgG$_{2a}$, 10 μg/ml) for an additional 20 min at 37°C. After another washing procedure, the β_2 integrins were stimulated by adding RAM (1:50 dilution). To facilitate the evaluation of PMA-induced effects, the dotted line in the figure shows the effect of β_2 integrin engagement alone (from Fig. 1). As a control, F-actin content was measured in cells treated with PMA alone (1 nM, n = 2, ◇). Samples were taken at the indicated times and the cellular F-actin content was determined. Data are given as mean ± SEM and expressed as percent of the time-zero value for each individual experiment.

stimulated neutrophils preincubated with 1 nM PMA for 10 min (Fig. 2), pretreatment with PMA at lower concentrations had no potentiating effect (data not shown). The response was twofold larger than that seen in β_2 integrin-stimulated but non-PMA–treated cells (Fig. 2). At this low concentration, PMA itself had no effect on the cellular F-actin content (Fig. 2). In contrast to PMA, the phorbol ester analogue 4-α-PMA, which does not activate protein kinase C, was unable to potentiate the β_2 integrin-induced F-actin response (Fig. 2).

Effect of Extracellular Calcium and Pertussis Toxin on the β_2 Integrin-stimulated F-actin Response

Removal of Ca²⁺ from the extracellular medium almost totally blocks actin polymerization induced by neutrophil adherence to a plastic surface whereas pertussis toxin has no effect (Southwick et al., 1989). However, in PMA-pretreated cells (Fig. 3, A), the β_2 integrin-induced F-actin response was only marginally affected (20-30% decrease), whereas the response for non-primed cells (Fig. 3 B) is reduced by half in the absence of extracellular Ca²⁺. In comparison, the fMLP-induced response is reduced by 20-30% when cells are stimulated in calcium-depleted medium (Fig. 3 C). In agreement with the results obtained from neutrophils adhering to a plastic surface, pertussis toxin pretreatment did not affect the β_2 integrin-induced F-actin response (142 ±

Figure 3. Effects of the removal of extracellular Ca²⁺ on β_2 integrin- and fMLP-induced actin polymerization. (A) Human neutrophils (6 × 10⁶ cells/ml) were incubated with PMA (1 nM) for 10 min at 37°C in either calcium-free (n = 3, ●) or calcium-containing (n = 3, ○) medium. After washing, the cells were resuspended in prewarmed calcium-free or calcium-containing medium and incubated with the IB4 antibody (anti-CD18, IgG$_{2a}$, 10 μg/ml) for an additional 20 min. The cells were then washed and resuspended in prewarmed volumes of their respective media, and the β_2 integrins were stimulated by addition of RAM (1:50 dilution). Just before stimulation, 1 mM EGTA was added to cells suspended in calcium-free medium to obtain a total depletion of free Ca²⁺ ions. Cells from the same donor preparation were used in paired experiments to minimize individual differences. (B) Stimulation of β_2 integrins on cells suspended in either calcium-free (n = 3, ■) or calcium-containing (n = 3, □) medium were carried out as described for A but without PMA pretreatment. Cells from the same donor preparation were used in paired experiments to minimize individual differences. (C) Cells (6 × 10⁶/ml) were incubated for 5 min at 37°C in either calcium-free (n = 3, ▲) or calcium-containing (n = 3, △) medium and then stimulated with fMLP (100 nM). Immediately prior to stimulation, 1 mM EGTA was added to cells suspended in calcium-free medium to obtain a total depletion of free Ca²⁺ ions. Cells from the same donor preparation were used in paired experiments to minimize individual differences. Samples were taken at the indicated times and the cellular F-actin content was determined. Data are given as mean ± SEM and expressed as percent of the time-zero value for each individual experiment.

10%, mean ± SEM, n = 4; 30 s after receptor engagement), compared with the response of nontreated control cells (139 ± 6%). In comparison, pertussis toxin totally abolished the fMLP-induced F-actin response (102 ± 4%, mean ± SEM, n = 3; 30 s after the addition of fMLP), compared with the response of nontreated control cells (254 ± 12%).
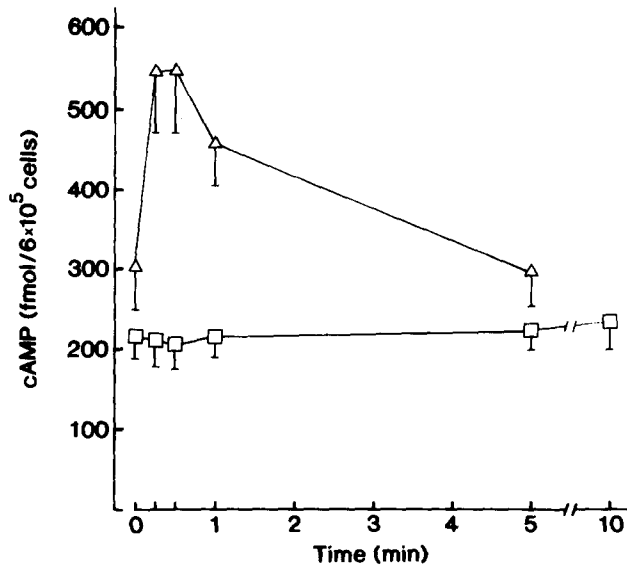
Figure 4. Effects of β_2 integrin engagement and stimulation with fMLP on the cellular content of cAMP. Human neutrophils (6 × 10^6 cells/ml) were incubated for 5 min at 37°C in calcium-containing medium and subsequently exposed to fMLP (100 nM; $n = 7$, △). Engagement of β_2 integrins was accomplished by first incubating the cells with the IB4 antibody (anti-CD18, IgG$_{2a}$, 10 μg/ml) for 20 min at 37°C and then, after washing, adding RAM (1:50 dilution, $n = 7$, □). At times indicated in the figure, aliquots of the cell suspensions were taken and the cellular content of cAMP was determined as described under Materials and Methods. Data are given as mean ± SEM and expressed as fmol/6 × 10^5 cells.

Effects of β_2 Integrin Engagement and Stimulation with fMLP on the Cellular Level of cAMP

Stimulation of the β_2 integrins under conditions which trigger actin polymerization did not affect the cellular cAMP level (Fig. 4), nor did the addition of the IB4 antibody per se alter the level of cAMP in unstimulated non-adherent cells (data not shown). This is in contrast to the chemotactic factor fMLP, which elicited a well-characterized transient increase in the cAMP level in non-adherent neutrophils (Fig. 4).

Effects of cAMP and IBMX on the β_2 Integrin-induced F-actin Response in Electropermeabilized Cells

To evaluate the absence of a cAMP signal on the β_2 integrin-induced F-actin response we added cAMP and IBMX (a phosphodiesterase inhibitor) to electropermeabilized neutrophils. The additions were made 30 s after β_2 integrin stimulation, since the F-actin response then reached its maximum (Fig. 2). We used 100 μM of both cAMP and IBMX, since it has previously been shown that this is a proper concentration when studying the effects of these substances on the F-actin content in electropermeabilized neutrophils (Downey et al., 1991). The permeabilized cells were divided into three equal portions referred to as the first, second, and third cell groups. RAM was added to all three cell groups to stimulate the β_2 integrins. Considering the cells of the first group (controls), to which no further additions were made, the F-actin response (Fig. 5, controls) was somewhat slower and of approximately half the magnitude of that
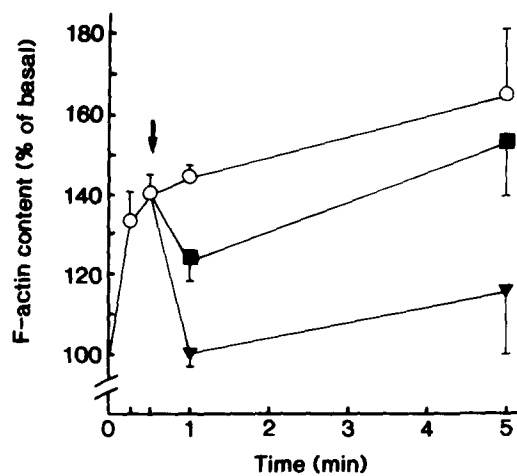

Figure 5. Effects of cAMP and IBMX on β_2 integrin-induced actin polymerization in electropermeabilized neutrophils. Human neutrophils (6 × 10^6 cells/ml) were incubated with PMA (1 nM) for 10 min at 37°C in calcium-containing medium. After washing, the cells were incubated with the IB4 antibody (anti-CD18, IgG$_{2a}$, 10 μg/ml) for 20 min; during the last 5 min of this incubation the cells were kept on ice. The cells were then washed and resuspended in the previously described permeabilization medium (1 × 10^7 cells/ml) and subsequently permeabilized by exposure to repeated electrical discharges (1.7 kV/cm). The entire permeabilization procedure was performed at 4°C. The cells were then further diluted with the permeabilization medium (6 × 10^6 cells/ml) and incubated for 5 min at 37°C. The suspension was then divided into three equal portions referred to as cell groups. RAM (1:50 dilution) was added to each of the groups to stimulate the β_2 integrins of the cells. The three groups were then respectively treated as follows: no further additions were made (control group, $n = 5$, o); cAMP (100 μM) was added ($n = 4$, ■); cAMP and IBMX (both 100 μM) were added simultaneously ($n = 3$, ▼). The arrow indicates time of additions. Data are given as mean ± SEM and expressed as percent of the time-zero value for each individual experiment.

seen in intact cells (Fig. 2). To the second group of cells cAMP (100 μM) was added immediately after the 30 s time point (Fig. 5, arrow), resulting in a prompt (within 30 s) decrease in the F-actin content; this inhibitory effect was partially reversed with time (5 min). To the third group, cAMP (100 μM) and IBMX (100 μM) were added, also immediately after the 30 s time point. The F-actin response in these cells was suppressed to basal levels within 30 s, and this inhibition was persistent.

Effects of β_2 Integrin Engagement and Stimulation with fMLP on the F-actin Response and the Formation of ^{32}P-PtdInsP$_3$

In neutrophils, fMLP induces both actin polymerization and PtdInsP$_3$ formation; these inductions have parallel time courses and dose responses (Eberle et al., 1990). Considering this, we investigated whether β_2 integrin engagement also leads to increased ^{32}P-PtdInsP$_3$ formation. We found that in neutrophils labeled with ^{32}P for 90 min, stimulation with the chemotactic factor fMLP resulted in a transient formation of ^{32}P-PtdInsP$_3$ (Fig. 6, inset) with a time course and magnitude very similar to those previously reported (Traynor-Kaplan et al., 1989; Eberle et al., 1990). Cells labeled in the same manner and then subjected to β_2 integrin stimu-
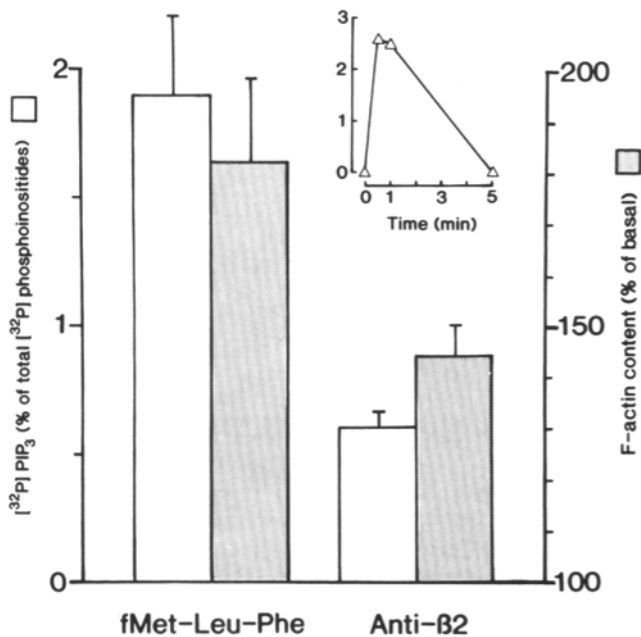
Figure 6. Effects of β_2 integrin engagement and stimulation with fMLP on the cellular content of F-actin and ^{32}P-PtdInsP$_3$. Human neutrophils (5 × 10^7 cells/ml) were incubated for 90 min with or without ^{32}P as described in Materials and Methods. During incubation, the IB4 antibody (anti-CD18, IgG$_{2a}$, 10 μg/ml) and PMA (1 nM, for the last 10 min) were added to the cells to be tested regarding the effect of β_2 integrin engagement; no supplements were added to the cells to be tested for the effect of fMLP. After washing, the cells were resuspended in calcium-containing medium to a concentration of 1.5 × 10^7/ml. The cells were then stimulated with fMLP (100 nM, n = 3) or their β_2 integrins were engaged by adding RAM (1:50 dilution, n = 3). Immediately before and 30 s after these additions the cellular content of F-actin (▨) and of ^{32}P-Ptd-InsP$_3$ (□) were determined as described in Materials and Methods. The data for F-actin and ^{32}P-PtdInsP$_3$ are given as mean ± SEM and expressed as percent of the cellular content of F-actin in unstimulated cells or of total ^{32}P-labeled phosphoinositides (PtdIns, PtdInsP, PtdInsP$_2$, PtdInsP$_3$), respectively. The inset illustrates the time course of fMLP (1 μM) induced formation of ^{32}P-PtdInsP$_3$ from one representative experiment.

lation did not produce detectable amounts of ^{32}P-PtdInsP$_3$, even if the cells had been pre-exposed to PMA (data not shown). It should be pointed out, however, that under these conditions β_2 integrin stimulation induced only a negligible F-actin response (data not shown). This response was far better preserved if the 90-min incubation required for ^{32}P-labeling was performed in the presence of the IB4 antibody and with PMA added during the last 10 min. Using this approach, β_2 integrin stimulation yielded a significant F-actin response, which was approximately half of the fMLP-induced response obtained after a 90-min pre-incubation (Fig. 6). To further improve the HPLC resolution, four samples of identically stimulated ^{32}P-labeled neutrophils were pooled before analysis. In unstimulated cells, no ^{32}P-PtdInsP$_3$ was detected, regardless of the ^{32}P-labeling procedure used. After β_2 integrin stimulation for 30 s, however, a distinct formation of ^{32}P-PtdInsP$_3$ was observed. This response was less than half of the response obtained after

fMLP stimulation for 30 s (Fig. 6). In accordance with fMLP stimulation, β_2 integrin engagement causes a rapid decrease in ^{32}P-PtdInsP$_2$ levels. β_2 integrin stimulation for 30 s decreased the ^{32}P-PtdInsP$_2$ level from 57.0 ± 0.6 to 51.3 ± 0.3 (data given as mean ± SEM, n = 3) per cent of total ^{32}P-labeled phosphoinositides (PtdIns, PtdInsP, PtdInsP$_2$, PtdInsP$_3$).

A possible effect of pertussis toxin on the β_2 integrin-induced formation of PtdInsP$_3$ formation was impossible to evaluate since the concentration of pertussis toxin needed during the ^{32}P-labeling procedure to abolish the subsequent fMLP-induced PtdInsP$_3$ formation (Traynor-Kaplan et al., 1989) unspecifically reduced the basal F-actin level by ∼50%. This is perhaps not so surprising since the concentration needed (10 μg/ml; Traynor-Kaplan et al., 1989) is 10–50 times that normally required to abolish agonist-induced effects mediated via a pertussis toxin sensitive heterotrimeric G-protein.

Discussion

This study demonstrates that direct engagement of β_2 integrins on non-adherent human neutrophils induces actin polymerization. The experiments showed that actin polymerization induced by β_2 integrin engagement was approximately half the magnitude of that induced by the chemotactic factor fMLP. Wright and Meyer (1986) have found that β_2 integrin (CR3) mediated phagocytosis (a process vitally dependent on modulations of the actin network) is potentiated by pretreatment with PMA, an agent that activates protein kinase C. Using a PMA pretreatment similar to that used by Wright and Meyer (1986), we obtained a twofold potentiation of the β_2 integrin-induced F-actin response. This effect was not, however, achieved by pretreatment with 4-α-PMA, a phorbol ester analogue that does not activate protein kinase C. Furthermore, Roubey et al. (1991) have shown that the effect of PMA pretreatment on β_2 integrin (CR3) mediated phagocytosis can be blocked by the protein kinase C inhibitor staurosporine. These findings indicate that the observed priming effects of PMA are probably mediated via protein kinase C. The response obtained after PMA addition is clearly not additive, since the PMA concentration used in the present experiments had no effect on the cellular content of F-actin. Consequently, it seems reasonable to suggest that the PMA pretreatment, at some level, positively modulates the β_2 integrin signaling system responsible for the effect on the actin network. It is possible that this effect is related to the ability of PMA to induce clustering of CD11b/CD18 on neutrophils (Detmers et al., 1987) and/or to a direct effect on the generation of the second messenger(s) involved in triggering actin polymerization.

The observed β_2 integrin-induced F-actin response can be compared with earlier findings that actin polymerization is elicited in neutrophils upon their adherence to a plastic surface (Southwick et al., 1989). The F-actin responses seen in these two studies appear to differ in sensitivity to removal of extracellular Ca^{2+}. More specifically, the actin polymerization induced by adherence to a plastic surface is almost totally abolished by removal of extracellular Ca^{2+} (Southwick et al., 1989), whereas the specific β_2 integrin-induced responses were only reduced by 20–30% or by half depend-

ing on whether or not the cells had been pre-exposed to PMA. This could suggest that antibody-induced clustering of β_2 integrins does not in all aspects mimic the adherence to a surface. However, in this context it should be noted that different surfaces cause distinct neutrophil responses. For example, neutrophil locomotion is dependent on intracellular Ca^{2+} signaling on a fibronectin- or vitronectin- but not on an albumin-coated surface (Marks and Maxfield, 1990; Marks et al., 1991). Consequently, the different requirements and cellular responses induced by differently coated surfaces seem to compare with the different anti-β_2 integrin-induced responses obtained from cells pretreated or not with PMA.

Considering the kinetics of the F-actin responses observed in the present study and that performed by Southwick et al. (1989), one common feature is apparent: both the specific β_2 integrin- and adhesion-induced increases in cellular F-actin content are relatively long-lasting, as compared with the increase induced by chemotactic factors. In contrast to the chemotactic peptide fMLP, β_2 integrin activation is not associated with an increase in cAMP content, however, direct addition of cAMP to electropermeabilized cells attenuated the β_2 integrin-induced F-actin response. In accordance, previous findings showed that cAMP induces depolymerization of F-actin in epithelial cells (Hays and Lindberg, 1991) and that elevated cAMP levels decrease the phagocytic capacity of neutrophils (Fällman et al., 1989). The absence of a cAMP signal might therefore, at least in part, be responsible for the prolonged β_2 integrin-induced F-actin response, that could be involved in the cell-stiffening (Downey et al., 1991) and the increased retention of neutrophils in the capillaries before their transendothelial migration (Doerschuk et al., 1987). Furthermore, these observations open up the possibility that the dynamic modulation of the actin network in moving neutrophils occurs as a result of a delicate second messenger cross-talk between β_2 integrin and chemotactic-factor receptors.

It has recently been suggested that an increased accumulation of diacylglycerol in the plasma membrane of *Dictyostelium discoideum* could, by a mechanism not dependent on protein kinase C, be the second messenger responsible for triggering polymerization of actin by increasing the actin nucleating activity (Shariff and Luna, 1992). Stimulation of neutrophils with a high concentration of PMA (100 nM) results in a major increase in the cellular mass of diacylglycerol (Fällman et al., 1989) and an increase in the cellular content of F-actin (Downey et al., 1992). In contrast, stimulation with fMLP triggers an increase in cellular F-actin levels to an even higher level (Downey et al., 1992), despite the fact that an increase in the cellular mass of diacylglycerol is hard to detect unless the cells are pretreated with cytochalasin B, an effective blocker of actin polymerization (Honeycutt and Niedel, 1986). Furthermore, engagement of β_2 integrins by an approach identical to that used to trigger actin polymerization in the present study does not lead to a significant increase in the cellular mass of diacylglycerol (unpublished data). These data raises questions regarding a cellular accumulation of diacylglycerol as a principal signaling event responsible for triggering actin polymerization in neutrophils.

The fact that β_2 integrin-induced F-actin response is re-duced by half by the 90-min incubation required for radio labeling of the phospholipids is in agreement with the previous observation that β_2 integrin-induced cytosolic free Ca^{2+} elevations are significantly reduced upon incubation at $37°C$ (Richter et al., 1990*b*). This is in contrast to fMLP-induced responses which if anything are potentiated by a similar preincubation. The observations that adding anti-CD18 antibodies or EGTA (data not shown) to cells subjected to prolonged incubation at a high cell density partially restored the β_2 integrin-induced F-actin response suggests that the desensitization of β_2 integrins could be due to micro-aggregations of the cells.

The proposed role for PtdIns(4,5)P$_2$ in promoting dynamic alterations of the actin network has been questioned by various groups (Bengtsson et al., 1988, 1990; Eberle et al., 1990; Dadabay et al., 1991). This led to studies of the PtdIns 3-kinase induced formation of PtdInsP$_3$ and its relations to chemotactic-factor-induced changes in the F-actin content in human neutrophils (Eberle et al., 1990). Based on the obtained results, PtdInsP$_3$ was suggested to participate in the regulation of actin polymerization (Eberle et al., 1990; Dobos et al., 1992). Further support for such a mechanism comes from a study by Zhang et al. (1992) showing that thrombin-induced activation of platelets cause an association between the PtdIns 3-kinase and the cytoskeleton. The present observations that the PtdInsP$_3$ response seen in β_2 integrin-stimulated cells is less than half of that seen in fMLP-stimulated cells and that a similar quantitative difference was noted for the ability of these two receptors to trigger actin polymerization are in line with the idea that PtdInsP$_3$ is related to receptor-induced actin polymerization. This idea is compatible with the original suggestion by Lassing and Lindberg (1985) that the phosphoinositide metabolism is involved in the regulation of the actin network via interaction with actin-binding proteins. The findings that PtdIns(4)P triggered actin polymerization but not as effectively as PtdIns(4,5)P$_2$ (Lassing and Lindberg, 1985) suggests that the higher degree of phosphorylation of PtdInsP$_3$ may give it a higher affinity for profilin and make it even more effective in triggering actin polymerization (Dobos et al., 1992).

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