Ca^{2+} signalling mechanisms of the β_2 integrin on neutrophils: involvement of phospholipase Cγ2 and Ins(1,4,5)P_3

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Engagement of β_2 integrins triggers a tyrosine kinase-dependent intracellular mobilization and influx of Ca^{2+} in human neutrophils. However, the transduction pathway involved in generating this Ca^{2+} signal is obscure. In the present study we identified phospholipase Cγ2 (PLCγ2) as one of the major proteins that was phosphorylated on tyrosine in response to β_2 integrin activation. This β_2 integrin-induced phosphorylation of PLCγ2 occurred in parallel with an increased accumulation of Ins(1,4,5)P_3. The relevance of these observations for the β_2 integrin-induced Ca^{2+} signal was investigated using an inhibitor of PLC signalling pathways, 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione (U73122). U73122 dose-dependently (IC_{50}, approx. 0.15 μM) inhibited both the β_2 integrin-induced release of Ca^{2+} from intracellular stores and the subsequent influx of Ca^{2+} across the plasma membrane. These effects were not observed with the inactive analogue 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-pyrrolidine-2,5-dione (U73343). To gain further support for an involvement of PLC-induced Ins(1,4,5)P_3 formation in the β_2 integrin-induced Ca^{2+} signal, we searched for the molecular event(s) underlying the effects of U73122. Our experiments revealed that U73122 had no effect on either β_2 integrin-induced tyrosine phosphorylation of PLCγ2 (or any of the other proteins) or on the formation of Ins(1,4,5)P_3, but it reduced the Ins(1,4,5)P_3-induced release of $^{45}Ca^{2+}$ from intracellular stores of electropermeabilized cells. Taken together, the present data suggest that the β_2 integrin-induced Ca^{2+} signal in human neutrophils is generated through activation of a PLCγ2-dependent pathway.

INTRODUCTION

Cell migration is a phenomenon that requires dynamic adhesive interactions between the internal motile machinery and the external substratum, with adhesion receptors such as integrins serving as transmembrane links. A good example of the importance of cell migration is the recruitment of neutrophils to the site of an inflammatory reaction. In this process, neutrophil adhesion via β_2 integrins is crucial for the cell–cell and cell–matrix interactions involved [1]. The β_2 integrins are non-covalently associated heterodimers composed of a common β-chain, CD18, and one of three unique α-chains, CD11a, CD11b or CD11c, with CD11b/CD18 being the most abundant β_2 integrin on neutrophils [2].

Adherent and migrating neutrophils exhibit spontaneous elevations in their cytosolic free Ca^{2+} concentration [3]. These Ca^{2+} signals are required for cell locomotion [4], possibly for the recycling of integrins to the front of the migrating neutrophil [5]. The Ca^{2+} signals can be blocked by pre-incubating the cells with antibodies directed against CD11b and/or CD18 [6,7], which implies that they are triggered by engagement of β_2 integrins. In further support of this possibility, the specific antibody engagement of CD11b or CD18 has been found to induce a transient increase in the cytosolic free Ca^{2+} concentration in neutrophils [8]. Moreover, it has been shown that the β_2 integrin-induced Ca^{2+} signal in neutrophils is due to both a release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} across the plasma membrane [8,9]. Although the β_2 integrin-induced release of intracellular Ca^{2+} is derived from thapsigargin-sensitive stores [10], the formation of Ins(1,4,5)P_3 connected with this release is small [11]. These data are compatible with a localized β_2 integrin-induced formation of Ins(1,4,5)P_3, which is indirectly supported by the observation that engagement of β_2 integrins induces a local release of Ca^{2+} from intracellular stores in neutrophils [9]. These results, along with our recent finding that tyrosine kinase activation precedes the release of Ca^{2+} from intracellular stores [12], indicate that clustering of integrins could be coupled to local activation of a tyrosine kinase(s) and a phospholipase C (PLC)γ (following its phosphorylation on tyrosine). Indeed, it has been reported that engagement of CD11a/CD18 integrins on T-lymphocytes induces tyrosine phosphorylation and activation of PLCγ1 [13]. However, this finding is not immediately applicable to neutrophils, since HL60 granulocytic cells were found to express the PLCγ2 isoform of the enzyme exclusively [14].

The aim of the present investigation was to explore the mechanism underlying the tyrosine kinase dependency of the β_2 integrin-induced Ca^{2+} signal in neutrophils.

EXPERIMENTAL

Chemicals

The chemicals used and their sources were as follows: 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione (U73122) and 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-pyrrolidine-2,5-dione (U73343), Biomol Research Lab. Inc. (Plymouth Meeting, PA, U.S.A.); PMSF, fMet-Leu-Phe, creatine kinase, creatine phosphate and DMSO, Sigma Chemical Co. (St. Louis, MO, U.S.A.); thapsigargin, LC Services Corp. (Woburn, MA, U.S.A.); ATP,

Abbreviations used: ECL, enhanced chemiluminescence; fura 2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid penta-acetoxymethyl ester; mAb, monoclonal antibody; PLC, phospholipase C; RAM, rabbit anti-mouse; U73122, 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione; U73343, 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-pyrrolidine-2,5-dione.

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Ins(1,4,5)P_3, oligomycin, antimycin A and ionomycin, Calbiochem–Behring Corp. AG (La Jolla, CA, U.S.A.); 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid/penta-acetoxymethyl ester (fura 2/AM), Molecular Probes Inc. (Eugene, OR, U.S.A.); enhanced chemiluminescence (ECL) kit, Ins(1,4,5)P_3 assay kit and $^{45}Ca^{2+}$, Amersham International (Amersham, Bucks., U.K.); leupeptin-O and pepstatin, Boehringer–Mannheim (Mannheim, Germany); Protein A-Sepharose, dextran and Ficoll–Hypaque, Pharmacia Fine Chemicals (Uppsala, Sweden); electrophoresis reagents, Bio-Rad (Richmond, CA, U.S.A.); nitrocellulose membrane, Schleicher and Schuell (Dassel, Germany). All other chemicals were of analytical grade.

Antibodies

The mouse monoclonal antibody (mAb), IB4 (IgG_{2a}), directed against the CD18 chain of the β_2 integrin was originally from Dr. S. Wright (Rockefeller University, New York, NY, U.S.A. [15]). The anti-phosphotyrosine mouse antibody (mAb clone 4G10; IgG_{2bk}) was from Upstate Biotech. Inc. (Lake Placid, NY, U.S.A.), the PLCγ2 antiserum used for immunoprecipitation was a gift from Dr. G. Carpenter (Vanderbilt University, Nashville, TN, U.S.A.) and the anti-PLCγ2 polyclonal antibody used for Western blot detection was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The rabbit anti-mouse (RAM) immunoglobulins were obtained from Dakopatts (Glostrup, Denmark), and the peroxidase-conjugated goat (anti-mouse) and goat (anti-rabbit) IgGs were obtained from Jackson ImmunoResearch Lab. Inc. (West Grove, PA, U.S.A.).

Preparation of human neutrophils

Blood from healthy volunteers was collected into heparin-containing tubes and the neutrophils were isolated according to a method decribed previously [16]. In short, after removal of erythrocytes by dextran sedimentation and brief hypotonic lysis, the cell suspension was centrifuged on a Ficoll-Hypaque gradient to separate the polymorphonuclear leucocytes from lymphocytes, monocytes and platelets. The resulting suspension, which contained approx. 98% granulocytes, was washed twice and resuspended in a Ca^{2+}-containing medium: 136 mM NaCl/4.7 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/5 mM $NaHCO_3$/20 mM Hepes/1.1 mM $CaCl_2$/0.1 mM EGTA/5.5 mM glucose (pH 7.4). Cells used for Western blot analysis and immunoprecipitation were routinely pretreated with 5 mM di-isopropyl fluorophosphate for 30 min at 4 °C.

Engagement of β_2 integrins

Neutrophils (5×10^6/ml) were incubated for 20 min at 37 °C in Ca^{2+}-containing medium supplemented with 10 μg/ml mouse mAb IB4 against CD18. After washing, the cells were resuspended in Ca^{2+}-containing medium or in Ca^{2+}-free medium (i.e. the Ca^{2+}-containing medium described above but without $CaCl_2$ and EGTA) and the β_2 integrins were engaged by the addition of RAM immunoglobulins (final dilution, 1:50). Control experiments were performed using $F(ab')_2$ fragments of both the mAb IB4 and the RAM immunoglobulins to exclude the possibility that any of the observed responses were due to Fc-receptor engagement. The results show that the $F(ab')_2$ fragments of both IB4 and RAM immunoglobulins elicited the same calcium-

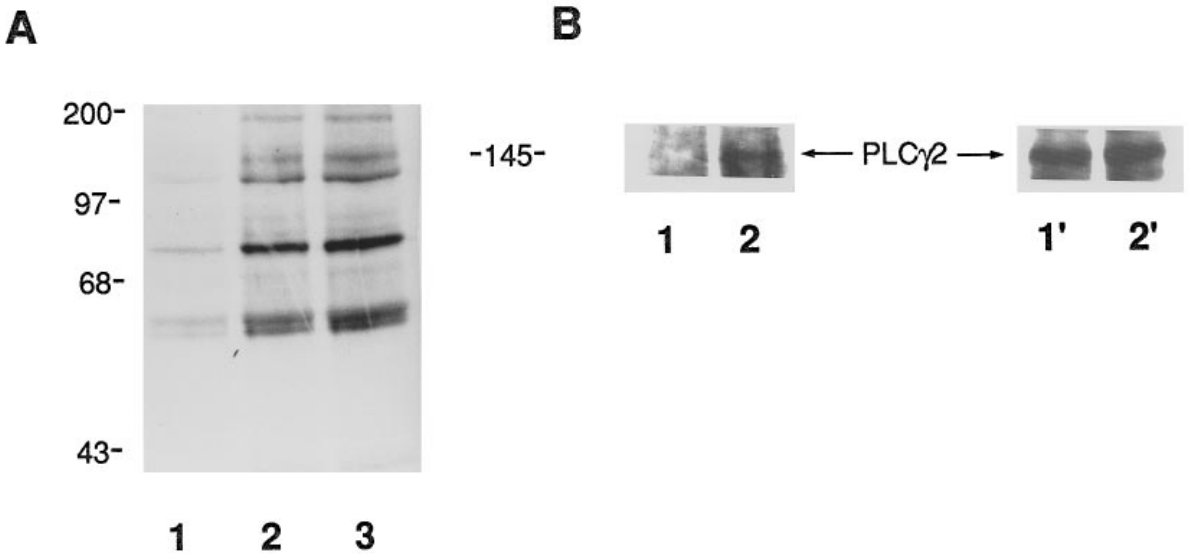

Figure 1 β_2 integrin-induced tyrosine phosphorylation of PLCγ2

(**A**) shows a Western blot of tyrosine-phosphorylated proteins from human neutrophils. Cells were incubated with mAb IB4 (10 μg/ml) in Ca^{2+}-containing medium for 20 min as described in the Experimental section and were incubated without any additive (lane 1) or with RAM antibodies for 30 s (1:50 dilution; lane 2) or 60 s (1:50 dilution; lane 3). The proteins resolved by electrophoresis were electrophoretically transferred to nitrocellulose membranes. Tyrosine-phosphorylated proteins were detected by using an anti-phosphotyrosine mAb (clone 4G10; 1:1000 dilution) and ECL. The blot is representative of at least three separate experiments. The numbers to the left of (**A**) indicate molecular mass (kDa). (**B**) shows a Western blot of tyrosine phosphorylation in immunoprecipitates of PLCγ2 from human neutrophils. Cells were incubated with mAb IB4 as described in the Experimental section, followed by incubation for 30 s without any additive (lanes 1 and 1′) or with RAM antibodies (1:50 dilution; lanes 2 and 2′). PLCγ2 was immunoprecipitated as described in the Experimental section and the proteins resolved by electrophoresis were electrophoretically transferred to a nitrocellulose membrane. The presence of tyrosine-phosphorylated proteins was detected using an anti-phosphotyrosine mAb (clone 4G10; 1:1000 dilution; lanes 1 and 2) and ECL. The blot was stripped and the presence of PLCγ2 was detected using anti-PLCγ2 polyclonal antibodies (1:500 dilution; lanes 1′ and 2′) and ECL. The immunoblots shown are representative of at least three separate experiments.

transient and protein-tyrosine-phosphorylation response as that of the intact antibodies.

Western blot analysis and immunoprecipitation

Neutrophils were suspended in Ca^{2+}-containing medium and were treated as described in the Figure legends. The reactions were stopped by transferring aliquots of cells to equal volumes of ice-cold Ca^{2+}-containing medium supplemented with 0.1 mM PMSF (final concentration) and were lysed as described previously [14]. Briefly, the cells were sedimented and resuspended in 100 μl of boiling lysis buffer containing 140 mM NaCl, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 50 mM Hepes (pH 7.5), 1 mM PMSF, 1 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 % (v/v) glycerol and 0.5 % (w/v) SDS. The resuspended samples were boiled for 5 min, diluted in ice-cold lysis buffer without SDS but containing 1 % (v/v) Nonidet P 40, sonicated and centrifuged at 13000 ***g*** for 10 min at 4 °C. The supernatants were used as whole-cell lysates. For immunoprecipitation, the supernatants were precleared once with Protein A–Sepharose beads and incubated for 2 h at 4 °C with 10 μl of anti-PLCγ2 antisera and 100 μl of a 50 % (v/v) slurry of Protein A–Sepharose beads with continuous rotation. The beads were then washed three times with 150 mM NaCl/50 mM Hepes (pH 7.5) and twice with 0.5 M LiCl/50 mM Hepes (pH 7.5). Finally, the precipitated proteins were boiled in Laemmli sample buffer. Electrophoresis was performed in SDS/7.5 % polyacrylamide gels as described by Laemmli [17] and the resolved proteins were electrophoretically transferred to nitrocellulose membranes. The presence of tyrosine-phosphorylated proteins or PLCγ2 were detected using specific monoclonal antibodies and ECL.

Determination of cytosolic free-calcium concentration

Neutrophils were loaded with the calcium indicator, fura 2, as described previously [18]. Briefly, 5×10^6 cells/ml were incubated for 20 min at 37 °C with 2 μM fura2/AM in Ca^{2+}-containing medium. For experiments involving β_2 integrin engagement, the mAb IB4 was present during the incubation. The cells were washed and resuspended in a cuvette containing 2 ml of the medium indicated in the respective Figure legends. Fluorescence was measured in a Spex spectrofluorimeter equipped with a thermostated (37 °C) cuvette holder and a continuous stirring device. Excitation wavelengths were 340 nm and 380 nm and the emission wavelength was 505 nm. Cytosolic free Ca^{2+} concentrations were calculated as described previously [19].

Formation of Ins(1,4,5)P_3

Neutrophils (2.5×10^7/ml) were incubated with mAb IB4 as described above. After stimulation with RAM immunoglobulin, the reaction was stopped by transferring 200 μl aliquots to an equal volume of ice-cold 15 % trichloroacetic acid. The samples were incubated on ice for 20 min and then centrifuged at 2000 ***g*** for 15 min at 4 °C. The supernatants were washed three times in water-saturated diethyl ether and stored at −20 °C. The amount of Ins(1,4,5)P_3 was determined using a commercial kit.

Intracellular release of $^{45}Ca^{2+}$ from electropermeabilized neutrophils

Neutrophils (2×10^7/ml) were suspended in 120 mM KCl/10 mM NaCl/2 mM $MgCl_2$/10 mM Pipes/3 mM EGTA/$CaCl_2$ containing 10 μM oligomycin, 10 μM antimycin A, 5 mM creatine phosphate and 5 units/ml creatine kinase. Calcium was buffered with EGTA to a theoretical concentration of 100 nM free Ca^{2+} by mixing equimolar solutions of EGTA and Ca-EGTA in proportions calculated using a computer program (Ligand) [20]. Aliquots (1 ml) of the cell suspension were exposed to repeated electrical discharges (150 μs each) of 1.7 kV/cm as described previously [21]. The cells were kept on ice during and after the permeabilization procedure and the degree of permeabilization was determined by Trypan Blue staining. The method used to study the intracellular release of $^{45}Ca^{2+}$ has been described elsewhere [22,23]. Briefly, cells were incubated at 37 °C with 6 μCi/ml $^{45}Ca^{2+}$ for 5 min and then for an additional 10 min in the presence of 1.5 mM ATP. During the last 3 min of the incubation period the cells were treated with 1 μM U73122 or with DMSO alone. The intracellular release of $^{45}Ca^{2+}$ was initiated by the addition of 2.5 μM Ins(1,4,5)P_3. The cellular content of $^{45}Ca^{2+}$ was determined by transferring 200 μl aliquots to 10 ml of ice-cold iso-osmotic sucrose buffer (310 mM sucrose/4 mM EGTA/5 mM Tris). The diluted samples were filtered immediately through glass-fibre filters (Whatman GF/C,

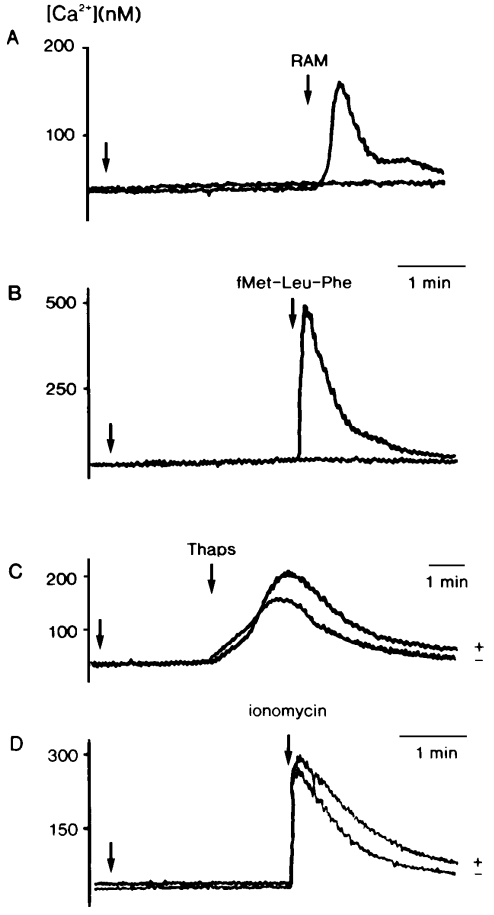

Figure 2 The effects of U73122 on the intracellular release of Ca^{2+} in human neutrophils

Neutrophils were loaded with fura 2 and, in experiments illustrated in (**A**), were also incubated with mAb IB4 (10 μg/ml) in Ca^{2+}-containing medium for 20 min, as described in the Experimental section. The cells were then washed and resuspended in 2 ml of Ca^{2+}-free medium supplemented with 1 mM EGTA, transferred to cuvettes, and subjected to fluorescence analysis at 37 °C. Where indicated (first arrow), 1 μM U73122 or DMSO alone was added to the cuvette. After preincubation for 3 min, the cells were stimulated (second arrow) by the addition of (**A**) RAM antibodies (1:50 dilution), (**B**) 100 nM fMet-Leu-Phe, (**C**) 2 μM thapsigargin (Thaps) or (**D**) 0.5 μM ionomycin. Each trace is representative of at least five identical experiments.

retention 1.2 μm) (pre-soaked in Ca^{2+}-containing medium) and then washed once with 10 ml of sucrose buffer. The radioactivity of each filter was subsequently determined.

RESULTS

β_2 integrin-induced phosphorylation of PLCγ2

We have previously shown that tyrosine kinase activation is a prerequisite for the β_2 integrin-induced Ca^{2+} signal [12]. In the present study, β_2 integrin engagement induced a rapid tyrosine phosphorylation of several proteins (Figure 1A). One of these migrated around 145 kDa and was shown to be PLCγ2 by immunoprecipitation of that particular protein, both before (Figure 1B, lane 1) and after (Figure 1B, lane 2) engagement of the β_2 integrins on neutrophils. The blot in Figure 1(B), which contained only one protein band, was stripped and reprobed with a different PLCγ2 antibody from that used in the immunoprecipitation procedure. This control experiment revealed equal amounts of PLCγ2 in both lanes of the nitrocellulose membrane (Figure 1B, lanes 1′ and 2′).

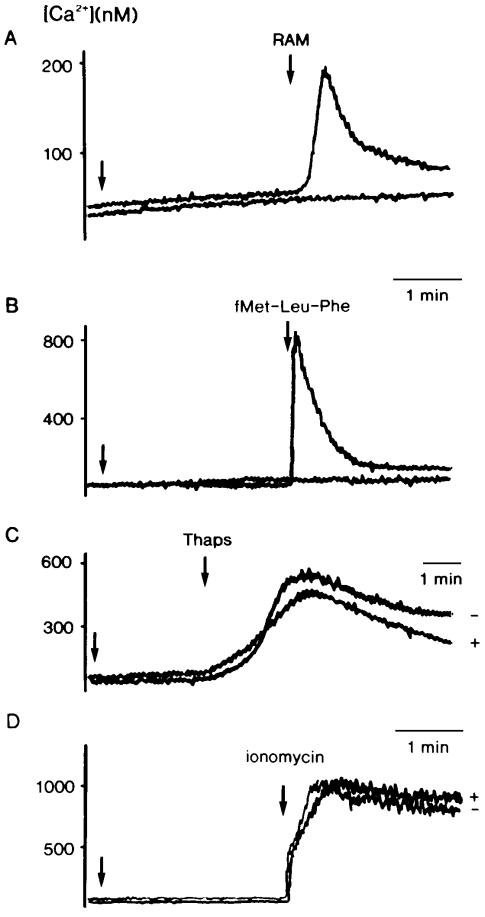

Figure 3 The effects of U73122 on the Ca^{2+} signal in human neutrophils

Neutrophils were loaded with fura 2 and, in the experiments illustrated in (**A**) also incubated with mAb IB4 (10 μg/ml) in Ca^{2+}-containing medium for 20 min, as described in the Experimental section. The cells were then washed and resuspended in 2 ml of Ca^{2+}-containing medium, transferred to cuvettes and subjected to fluorescence analysis at 37 °C. Where indicated (first arrow), 1 μM U73122 or DMSO alone was added to the cuvette. After preincubation for 3 min, the cells were stimulated (second arrow) by the addition of (**A**) RAM antibodies (1:50 dilution), (**B**) 100 nM fMet-Leu-Phe, (**C**) 2 μM thapsigargin (Thaps) or (**D**) 0.5 μM ionomycin. Each trace is representative of at least five identical experiments.

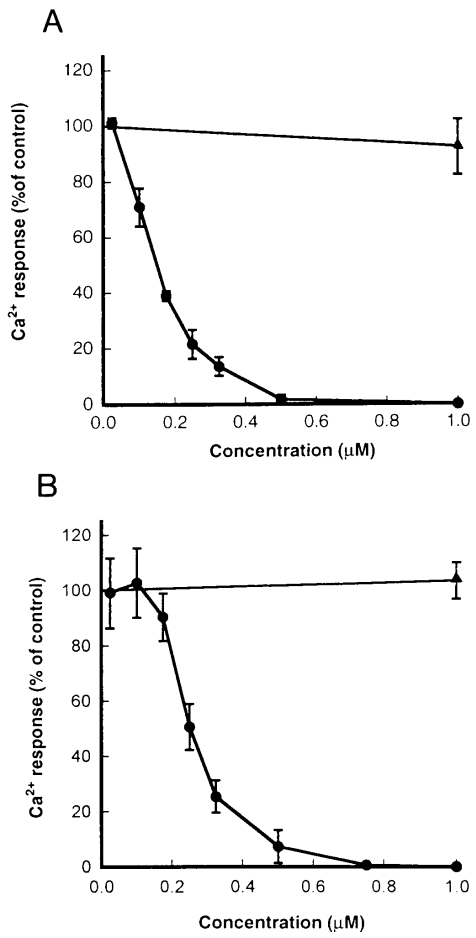

Figure 4 Dose-dependency of the effects of U73122 on receptor-induced Ca^{2+} signals in human neutrophils

Neutrophils were loaded with fura 2 and, in the experiments illustrated in (**A**) were also incubated with mAb IB4 (10 μg/ml) in Ca^{2+}-containing medium for 20 min, as described in the Experimental section. The cells were then washed and resuspended in 2 ml of Ca^{2+}-containing medium, transferred to cuvettes, and subjected to fluorescence analysis at 37 °C. Thereafter, the cells were preincubated for 3 min with the indicated concentrations of U73122 (●) and U73343 (▲). The cells were subsequently stimulated by the addition of (**A**) RAM antibodies (1:50 dilution) or (**B**) 100 nM fMet-Leu-Phe. The degree of inhibition induced by U73122 or U73343 was calculated as a percentage of the Ca^{2+} peak obtained in cells exposed to DMSO alone. The data given are the means ± S.E.M. of 3–4 separate experiments.

Effects of U73122 on agonist-induced Ca^{2+} signalling

To determine whether the β_2 integrin-induced Ca^{2+} signal is dependent on PLCγ2 activity, we tested the effects of U73122, a membrane-permeable aminosteroid that inhibits other PLC-dependent pathways in platelets and neutrophils [24,25]. To study the effect of U73122 on the release of Ca^{2+} from intracellular stores, cells were stimulated in a Ca^{2+}-depleted medium (Ca^{2+}-free medium supplemented with 1 mM EGTA). In control cells, engagement of β_2 integrins induced a rapid and transient release of Ca^{2+} from intracellular stores (Figure 2A, top trace). Pretreatment of cells with 1 μM U73122 for 3 min did not affect the intracellular Ca^{2+} concentration, but completely inhibited the intracellular release of Ca^{2+} induced by β_2 integrin engagement (Figure 2A, lower trace). The fMet-Leu-Phe-induced intracellular release of Ca^{2+} is known to be mediated by activation of PLCβ [26], hence we used this signal as a control and found that it was also completely blocked by pretreatment with 1μM U73122 for

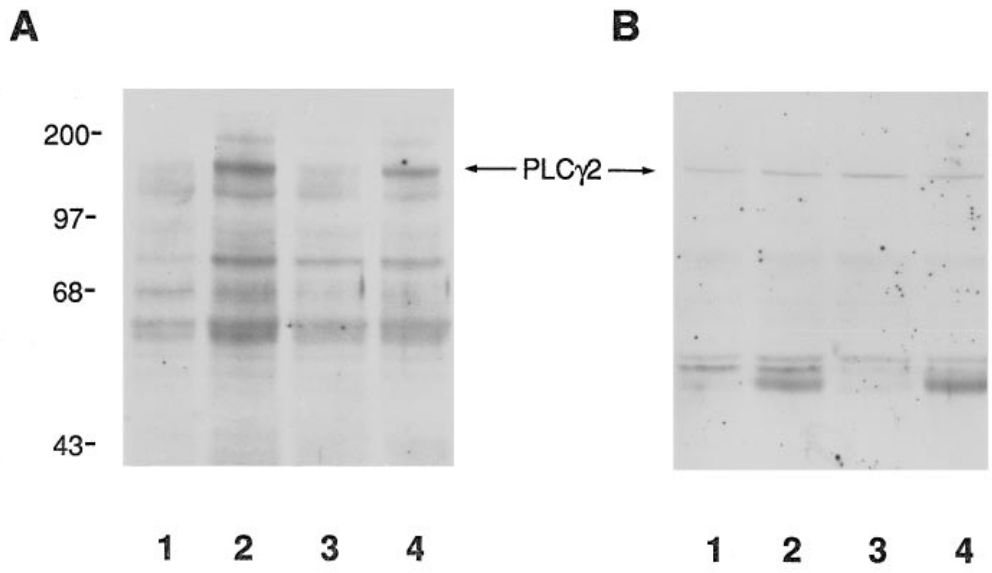

Figure 5 The effect of U73122 on the β_2 integrin-induced protein tyrosine phosphorylation in human neutrophils

In (**A**), neutrophils were incubated with mAb IB4 (10 μg/ml) in Ca^{2+}-containing medium for 20 min, as described in the Experimental section. The cells were then washed and resuspended in Ca^{2+}-containing medium, followed by incubation for 3 min with DMSO alone (lanes 1 and 2) or 1 μM U73122 (lanes 3 and 4) at 37 °C. The cells were then incubated for 30 s without any additives (lanes 1 and 3) or the β_2 integrins were engaged by the addition of RAM antibodies (1:50 dilution, lanes 2 and 4). The proteins resolved by electrophoresis were electrophoretically transferred to nitrocellulose membranes. Tyrosine-phosphorylated proteins were detected with an anti-phosphotyrosine mAb (clone 4G10, 1:1000 dilution) and ECL. The immunoblot is representative of at least three separate experiments. The numbers to the left of panel (**A**) indicate molecular mass (kDa). In (**B**); the blot was stripped and the presence of PLCγ2 was detected by using anti-PLCγ2 polyclonal antibodies (1:500 dilution) and ECL. The immunoblot is representative of at least three separate experiments.

3 min (Figure 2B). As PLC-independent controls, we used thapsigargin, which depletes intracellular Ca^{2+} stores by inhibiting Ca^{2+}-ATPase [27], and ionomycin. Pretreatment of the cells with 1 μM U73122 for 3 min did not affect the intracellular release of Ca^{2+} induced by either thapsigargin (Figure 2C) or ionomycin (Figure 2D). The small difference between the Ca^{2+} traces in Figure 2(C) merely reflects normal variations seen in the thapsigargin-induced rise in cytosolic free-Ca^{2+} concentration.

To study the effects of U73122 on the influx of Ca^{2+} across the plasma membrane, we repeated the experiments in the presence of extracellular Ca^{2+} (1 mM). Pretreatment of neutrophils with 1μM U73122 totally inhibited the Ca^{2+} signal induced by β_2 integrins (Figure 3A, lower trace) and by fMet-Leu-Phe (Figure 3B, lower trace). As expected, the Ins(1,4,5)P_3-independent Ca^{2+} signals induced by thapsigargin (Figure 3C) or ionomycin (Figure 3D) were not affected by U73122. The fMet-Leu-Phe-induced Ca^{2+} influx is considered to be triggered by an Ins(1,4,5)P_3-induced depletion of intracellular Ca^{2+} stores, and the present results suggest that the β_2 integrin-induced Ca^{2+} signal might be mediated via similar mechanisms.

The dose-dependency of the effect of U73122 on both the β_2 integrin- and fMet-Leu-Phe-induced Ca^{2+} signal was also determined, revealing IC_{50} values of approx. 0.15 μM (Figure 4A) and 0.25 μM (Figure 4B) respectively. U73343, an inactive analogue of U73122, did not affect the receptor-induced Ca^{2+} signals, even at the highest concentration used (1 μM; Figure 4).

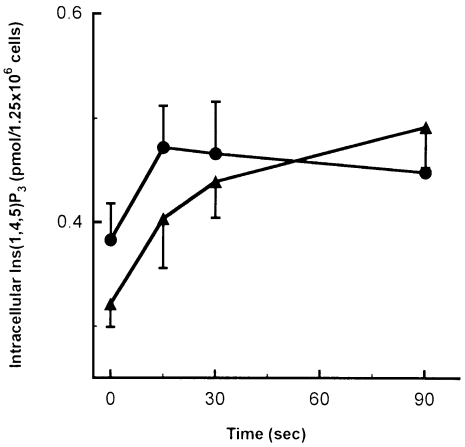

Figure 6 The effects of U73122 on the β_2 integrin-induced formation of Ins(1,4,5)P_3 in human neutrophils

Neutrophils were incubated with mAb IB4 (10 μg/ml) in Ca^{2+}-containing medium for 20 min, as described in the Experimental section. The washed cells were resuspended in Ca^{2+}-containing medium and incubated for 3 min with 1 μM U73122 (▲) or DMSO alone (●) at 37 °C. The β_2 integrins were engaged by the addition of RAM antibodies (1:50 dilution), the reactions were terminated at the times indicated and the concentration of Ins(1,4,5)P_3 was determined as described in the Experimental section. The amount of Ins(1,4,5)P_3 was calculated in pmol/1.25 $\times$ 10^6 cells and the data shown are the means $\pm$ S.E.M. of 5–7 separate experiments.

Effects of U73122 on β_2 integrin-induced activation of PLCγ2 and formation of Ins(1,4,5)P_3

The tyrosine phosphorylation of the 145 kDa band, identified as PLCγ2, was not affected by U73122 (compare lanes 2 and 4 in Figure 5A); this was verified by reprobing the blot in Figure 5(A) with an antibody directed towards PLCγ2 to confirm that lane 4 did not contain more protein than lane 2 (Figure 5B). The observation that tyrosine phosphorylation of PLCγ2 was

Table 1 Effect of U73122 on the Ins(1,4,5)P_3-induced intracellular release of $^{45}Ca^{2+}$ in electropermeabilized neutrophils

The intracellular release of $^{45}Ca^{2+}$ was measured as described in the Experimental section. The Ins(1,4,5)P_3-induced release of $^{45}Ca^{2+}$ is expressed as the difference (c.p.m.) between the content of $^{45}Ca^{2+}$ in untreated cells and the amount remaining in cells treated with Ins(1,4,5)P_3; the values given are the means ± S.E.M. of seven separate experiments; $P < 0.05$.

Pretreatment	$^{45}Ca^{2+}$ released (c.p.m.)
DMSO	3073 ± 379
U73122	1985 ± 289

unaffected by U73122 prompted us to search for the site of action of this inhibitor of PLC-dependent signalling pathways.

To investigate if the inhibition of the Ca^{2+} signal by U73122 resulted in decreased PLCγ2 activity, we studied the effect of this aminosteroid on the β_2 integrin-induced formation of Ins(1,4,5)P_3. The results outlined in Figure 6 clearly show that the relatively modest formation of Ins(1,4,5)P_3 in neutrophils was not affected by pretreatment with 1 μM U73122. Consequently, the effect of U73122 on β_2 integrin-induced Ca^{2+} signalling cannot be due to direct inhibition of PLCγ2.

Effects of U73122 on the Ins(1,4,5)P_3-induced release of Ca^{2+} in electropermeabilized neutrophils

To test the possibility that U73122 acts on Ins(1,4,5)P_3-induced mobilization of Ca^{2+} from intracellular stores, electropermeabilized neutrophils were preloaded with $^{45}Ca^{2+}$, exposed to Ins(1,4,5)P_3 and the release of $^{45}Ca^{2+}$ was determined. A significant quantity of $^{45}Ca^{2+}$ was released from the intracellular stores of control cells, whereas the addition of Ins(1,4,5)P_3 liberated only two-thirds as much from cells pretreated with 1 μM U73122 (Table 1).

DISCUSSION

The present study reveals that engagement of β_2 integrins triggers tyrosine phosphorylation of PLCγ2 and formation of Ins(1,4,5)P_3, suggesting that activation of this isoenzyme is crucial for the generation of a Ca^{2+} signal by β_2 integrins. Kanner et al. [13] found that engagement of β_2 integrins on T-lymphocytes induced tyrosine phosphorylation and activation of PLCγ1. Although granulocytic cells primarily express PLCγ2 [14], the results of both the present study and the findings of Kanner and co-workers [13] imply that the Ca^{2+}-signal pathway of integrins preferentially involves the γ-isoform of PLC. This isoenzyme of PLC is advantageous for integrin signalling because it contains *src*-homology (SH2 and SH3) domains that can interact with tyrosine-phosphorylated and/or cytoskeletal proteins, both of which co-localize with integrins to cellular focal adhesions [28]. Although non-phosphorylated and phosphorylated PLCγ exhibit similar catalytic activities *in vitro* [29], tyrosine phosphorylation appears to be necessary for its activation *in vivo* [30]. Consequently, if PLCγ2 does play a role in the β_2 integrin-induced Ca^{2+} signal in neutrophils, activation of one or more non-receptor tyrosine kinases is probably included in this signalling pathway.

Berton et al. [31] have reported that β_2 integrin-dependent adhesion triggers activation of the *fgr* tyrosine kinase, which belongs to the *src*-kinase family. This is relevant to our data because PLCγs are very good substrates, *in vitro*, for members of the *src*-kinase family. In addition, electroporation of *src*-kinase antibodies inhibits angiotensin-induced phosphorylation and activation of PLCγ1 in smooth-muscle cells [32]. Taken together, these observations infer that PLCγ may be a substrate for *fgr* tyrosine kinase after activation of β_2 integrins. Although the mechanism underlying the interaction between β_2 integrins and a non-receptor tyrosine kinase is not yet known, we recently demonstrated that activation of a tyrosine kinase precedes, and is a requirement for generation of subsequent β_2 integrin-induced Ca^{2+} signalling [12], and that β_2 integrin engagement induces tyrosine phosphorylation of PLCγ2 (present study). In this context, it should be mentioned that the specific tyrosine kinase $pp125^{FAK}$, a prime non-receptor tyrosine kinase that initiates β_1 and β_3 integrin-signalling events [33–36], has not been detected as one of the major tyrosine-phosphorylated protein bands after engagement of β_2 integrins on T-lymphocytes [13], HL60 granulocytic cells [12] or neutrophils. However, Schaller et al. [37] have recently demonstrated that β_1 integrin signal-transduction pathways can diverge, using either $pp125^{FAK}$ or paxillin (a cytoskeletal protein that can also interact with members of the *src*-kinase family). This work demonstrated that there is at least one alternative integrin signalling pathway that does not use $pp125^{FAK}$. Although the exact coupling between β_2 integrins and one or more tyrosine kinases is unclear, the present data suggest that the β_2 integrin-induced Ca^{2+} signal could be initiated by a tyrosine kinase-dependent formation of Ins(1,4,5)P_3 and subsequent mobilization of intracellular Ca^{2+}.

In neutrophils, Fc receptor engagement has been shown to cause tyrosine phosphorylation of PLCγ2 [38]; however, it has also been demonstrated that Fc receptor-induced mobilization of intracellular Ca^{2+} is not dependent on PLC-mediated Ins(1,4,5)P_3 formation [39]. Although the β_2 integrin-induced mobilization of Ca^{2+} is derived from thapsigargin-sensitive stores [10], we wanted to further assess whether or not the β_2 integrin-induced Ca^{2+} signal is mediated via PLC-mediated Ins(1,4,5)P_3 formation. Therefore cells were pretreated with the membrane-permeable aminosteroid U73122, a reported inhibitor of the PLC pathway [24,25]. Pretreatment of the cells with U73122 (1 μM), but not its inactive analogue U73343, totally inhibited the β_2 integrin-induced release of Ca^{2+} from intracellular stores as well as the influx across the plasma membrane. However, U73122 did not inhibit either the β_2 integrin-induced tyrosine phosphorylation of PLCγ2 or the formation of Ins(1,4,5)P_3. This is in agreement with the findings of Smith et al. [25], that U73122 inhibits fMet-Leu-Phe-induced formation of Ins(1,4,5)P_3 with an IC_{50} of about 2 μM, in comparison with the IC_{50} for the cytosolic free Ca^{2+} signal of around 0.5 μM. Furthermore, although fMet-Leu-Phe and β_2 integrins activate two different PLC isoforms ([13,26]; present results), U73122 inhibited the Ca^{2+} signal induced by both of these stimuli.

Therefore, if U73122 is an inhibitor of the PLC-signalling pathways, it must act downstream of PLC-induced Ins(1,4,5)P_3 formation. Using electropermeabilized neutrophils we demonstrated that U73122 could impair the ability of Ins(1,4,5)P_3 to mobilize intracellular Ca^{2+}. This may have been due to a direct effect of U73122 on the Ins(1,4,5)P_3-mediated release of Ca^{2+} or to a thapsigargin-like effect of U73122. The latter seems unlikely, since the concentration of U73122 used in the present study (1 μM) did not affect either the basal intracellular Ca^{2+} concentration or the thapsigargin-induced release or influx of Ca^{2+}. The fact that U73122 did not totally reduce the intracellular release of Ca^{2+} induced by Ins(1,4,5)P_3 in electropermeabilized cells could be explained, at least in part, by the requirement for a higher concentration of Ins(1,4,5)P_3 than was actually formed upon receptor engagement in this experimental procedure [40].

The present results clearly show that in neutrophils, at least, U73122 is not a specific PLC inhibitor.

In summary, the present available data lend further support to the idea that tyrosine phosphorylation and activation of PLCγ2 by β_2 integrins precede the generation of a Ca^{2+} signal. Our results suggest a possible explanation for the fact that U73122 inhibited the Ca^{2+} signal induced by receptors that activate two different isoforms of PLC, and provide evidence for a direct role of Ins(1,4,5)P_3 in the generation of the β_2 integrin-induced Ca^{2+} signal.

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