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Involvement of GTP-binding proteins in actin polymerization in human neutrophils

(signal transduction/aluminium tetrafluoride/guanine nucleotides/cytoskeleton dynamics/cell motility)

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ABSTRACT **The motility of human neutrophils, which is of vital importance for the role of these cells in host defense, is based on rapid and dynamic changes of the filamentous actin (F-actin) network. Consequently, to understand how neutrophils move and ingest particles, we need to know how polymerization and depolymerization of actin are regulated. Previous studies by several investigators have, based on indirect evidence obtained with pertussis toxin, suggested a role for GTP-binding protein(s) (G protein) in chemotaxis-induced, but not phagocytosis-induced, reorganization of the F-actin network. The aim of the present investigation was to study the effects of directly activated G proteins (i.e., without prior ligand–receptor complex formation) on the F-actin content in human neutrophils. AlF_4^- induced a pronounced and sustained increase in F-actin in intact neutrophils. This effect coincided with an increase in cytosolic free Ca^{2+}, indicating that phospholipase C and the subsequent transduction mechanism were also activated. Inhibition of phospholipase C activity by extensive depression of the cytosolic free Ca^{2+} level (< 20 nM) only marginally affected the AlF_4^--induced rise in F-actin content. The major part of the AlF_4^--induced rise in F-actin content was also resistant to pertussis toxin, suggesting that pertussis toxin-insensitive G proteins in neutrophils are also able to trigger actin polymerization. The specificity of AlF_4^- in activating G proteins was also tested in permeabilized cells. In this case the effect was more rapid and could be totally abolished by guanosine 5′-[β-thio]diphosphate. In analogy, in permeabilized cells guanosine 5′-[γ-thio]triphosphate mimicked the effect of AlF_4^- on actin polymerization, and the effect induced by this nonhydrolyzable GTP analogue could also be totally abolished by guanosine 5′-[β-thio]diphosphate. In summary, the present data support our previous hypothesis that G proteins are intimately linked to actin polymerization in human neutrophils.**

Accumulation of neutrophils at sites of infection and the subsequent destruction of invading microorganisms are regulated by extensive rearrangements of the peripheral cytoskeletal system (1). It is well established that actin, the major component of this system, is rapidly and transiently transferred from a globular (G-actin) to a filamentous (F-actin) form after contact with chemotactic as well as phagocytic stimuli (2, 3). Despite intensive research, the transducing signal(s) related to the initiation of actin polymerization is still inadequately understood.

In neutrophils, transmembrane signaling has been studied in great detail for the chemotactic peptide formylmethionylleucylphenylalanine (fMet-Leu-Phe). The signaling includes a GTP-binding protein (G protein) and the subsequent stimulation of a membrane-bound phospholipase C (4–8). Recently, it was shown that phagocytosis triggers the accumulation of both inositol trisphophate and diacylglycerol in human neutrophils (9, 10). Consequently, both chemotaxis- and phagocytosis-induced signaling seem to apply to the general idea of how a large number of different types of ligands generate second messengers via the phosphatidylinositol cycle (8).

Pertussis toxin, which catalyzes ADP-ribosylation leading to inactivation of the α subunit of G proteins, has been a useful tool in elucidating a role of G proteins and phospholipase C in the activation of various cell functions (11). We have shown (12), as have other authors (13), that fMet-Leu-Phe-induced actin polymerization is totally abolished by pretreating human neutrophils with pertussis toxin. This finding, together with the more recent observation that fMet-Leu-Phe-induced actin polymerization is independent of a preceding or concomitant activation of phospholipase C (14), indirectly suggests that G proteins could have a more direct role in the initiation of actin polymerization. However, it should be pointed out that receptor-mediated phagocytosis in human neutrophils, a process with an obvious requirement for actin polymerization, is only marginally affected by pertussis toxin (10, 15).

Fluoride is considered to be a general activator of G proteins in intact cells (16). This action probably requires aluminium ions, giving the active species AlF_4^- (17, 18). It is believed that AlF_4^- becomes associated with GDP molecules bound to the α subunits of nonactivated G proteins. This stable AlF_4^-/GDP complex mimics the effect of GTP, leading to a dissociation and persistent activation of G proteins (18). However, AlF_4^- has been shown to affect other cellular structures and systems, including the stability of F-actin (19) and the activity of Na^+,K^+-ATPase (20) and glucose-6-phosphatase (21). Consequently, it is important to activate G proteins by an alternative mechanism, for example, by adding the nonhydrolyzable GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[γS]) to permeabilized cells. In addition, the specific actions of AlF_4^- and GTP[γS] on G proteins can be confirmed by replacing bound GDP with its stable analogue guanosine 5′-[β-thio]diphosphate (GDP[βS]), thereby preventing the activation of G proteins (22). In human neutrophils, AlF_4^- has been shown to induce several biochemical and functional responses, including activation of phospholipase C and protein kinase C, increase in cytosolic free Ca^{2+}, release of arachidonic acid, and production of superoxide anion (23–28).

The aim of the present study was to bypass ligand–receptor complex formation and activate cellular G proteins with either AlF_4^- or GTP[γS], under various conditions, in order to assess the involvement of G proteins in the initiation of

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Abbreviations: F-actin, filamentous actin; G protein, GTP-binding protein; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate; CCM, calcium-containing medium; CFM, calcium-free medium; AM, acetoxymethyl ester.

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actin polymerization in human neutrophils. These data were presented in an abstract last year (29).

MATERIALS AND METHODS

Materials. The materials and their sources were as follows: dextran and Ficoll-Paque (Pharmacia); GTP[γS] and GDP-[βS] (Boehringer Mannheim); fluorescein-phalloidin (Molecular Probes); quin-2, quin-2 AM, fura-2, and fura-2 AM (where AM is acetoxymethyl ester) (Amersham); pertussis toxin (List Biological Laboratories, Campbell, CA).

Methods. Polymorphonuclear leukocytes (neutrophils) were prepared from peripheral human blood, essentially as described by Böyum (30), and then suspended (10^7 per ml) in a "calcium-containing medium" (CCM) with the following composition: 136 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/1.1 mM $CaCl_2$/0.1 mM EGTA/1.2 mM KH_2PO_4/5.0 mM $NaHCO_3$/5.5 mM glucose/20 mM Hepes, pH 7.4.

We previously demonstrated (10, 14) that the activity of phospholipase C could be inhibited in human neutrophils by reducing the level of cytosolic free Ca^{2+} with quin-2. In short, neutrophils suspended (10^7 per ml) in a "calcium-free medium" (CFM: the same as CCM but lacking $CaCl_2$ and supplemented with 1 mM EGTA) were incubated with 50 μM quin-2 AM for 60 min at 37°C. At the end of the incubation the cells were washed twice with and resuspended in CFM.

F-actin content was determined by staining with fluorescein-phalloidin, as described (14).

Cytosolic free calcium levels were monitored with the fluorescent probes quin-2 and fura-2, essentially as described (31–33).

Human neutrophils were electrically permeabilized according to a published method (34). In brief, the cells were suspended (10^7 per ml) in an ice-cold permeabilization medium: 20 mM NaCl/100 mM KCl/1 mM NaH_2PO_4/25 mM $NaHCO_3$/20 mM Hepes/1 mM EGTA/0.2% bovine serum albumin, pH 7.0. Aliquots (1 ml) of this preparation were exposed to 25 discharges of 1.7 kV/cm (150 msec each). During and after the permeabilization procedure, the neutrophils were kept on ice. The degree of permeabilization of the cells was routinely estimated by staining with trypan blue (6.25 mg/ml) for 10 min at 4°C, followed by microscopic examination (34).

RESULTS

Fluoride-Induced Effects on the F-Actin Content in Intact Cells. Addition of 20 mM NaF and 10 μM $AlCl_3$ (hereafter referred to as AlF_4^-) caused a slow but sustained increase in the cellular content of F-actin (Fig. 1). The response exhibited a typical lag period of about 5 min and reached its maximum (>300% of basal) after ≈40 min. Morphological examination after 40 min of stimulation revealed formation of pseudopodlike structures and a peripheral distribution of F-actin (see Fig. 5*B*). Cells preincubated with pertussis toxin (400 ng/ml), which totally inhibits chemotatic peptide-induced actin polymerization (12, 13), exhibited an almost unaffected increase in F-actin content in response to AlF_4^- (Fig. 1). Even when the cells were pretreated with extremely high concentrations of the toxin (up to 1600 ng/ml), the major part of the AlF_4^--induced increase in F-actin still occurred (Fig. 1, *Inset*). The role of phospholipase C activation was studied by lowering the concentration of cytosolic free Ca^{2+} to <20 nM (Fig. 2), a level previously shown to totally depress phospholipase C activity induced by AlF_4^-, by fMet-Leu-Phe, or by particles opsonized with complement component C3bi (10, 14, 28). The AlF_4^--induced increase in F-actin content was, however, only marginally reduced under these conditions (Fig. 1).

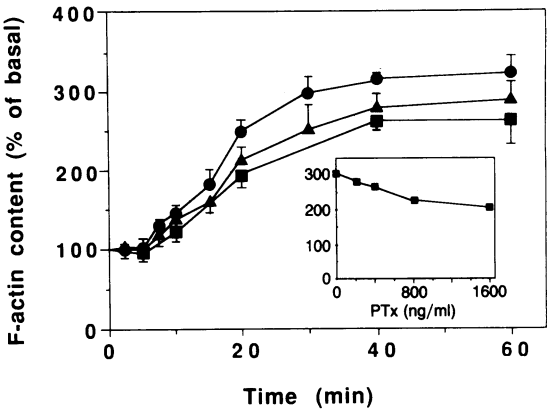

FIG. 1. Effect of AlF_4^- on the F-actin content in intact human neutrophils. The kinetics of the responses to stimulation with 20 mM NaF and 10 μM $AlCl_3$ under various conditions are shown. Before stimulation the different groups of cells (10^7 per ml) were treated as follows: incubated for 2 hr at 37°C in CCM (●; n = 8); loaded with quin-2 (50 μM) for 1 hr at 37°C in CFM, washed, and resuspended for 1 hr at 37°C in CFM (▲; n = 5); or pretreated with pertussis toxin (400 ng/ml) for 2 hr, washed, and resuspended for 5 min at 37°C in CCM (■; n = 5). Results are presented as means ± SEM of n experiments. (*Inset*) F-actin content in neutrophils preincubated for 2 hr with various concentrations of pertussis toxin (PTx), washed, resuspended in CCM, and then stimulated with 20 mM NaF and 10 μM $AlCl_3$ for 40 min; results are presented as means of two separate experiments.

Fluoride-Induced Changes in Cytosolic Free Ca^{2+}. AlF_4^--induced changes in cytosolic free Ca^{2+} levels were monitored spectrofluorometrically in fura-2- or quin-2-loaded neutrophils. After a lag time of 45–60 sec, AlF_4^- caused a slow but steady rise in cytosolic free Ca^{2+}, from about 130 nM to a maximum of ≈500 nM, within 20 min (Fig. 2). The AlF_4^--stimulated rise in cytosolic free Ca^{2+} was not significantly affected by pertussis toxin (400 ng/ml; Fig. 2), which is consistent with the lack of effect of the toxin on the AlF_4^--induced increase in F-actin content (Fig. 1). Loading cells with 50 μM quin-2 in CFM reduced their resting Ca^{2+} level

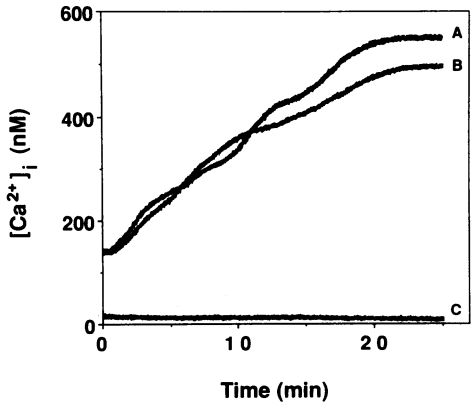

FIG. 2. Effect of AlF_4^- on the level of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in intact human neutrophils. The tracings shown are fluorometric recordings of fura-2- or quin-2-loaded neutrophils after stimulation (at time zero) with 20 mM NaF and 10 μM $AlCl_3$. Before stimulation the different groups of cells (10^7 per ml) were treated as follows: incubated for 2 hr at 37°C in CCM, exposed to 2 μM fura-2 AM during the last 30 min, washed, and resuspended for 5 min at 37°C in CCM (trace A); pretreated with pertussis toxin (400 ng/ml) for 2 hr at 37°C in CCM, exposed to 2 μM fura-2 AM during the last 30 min, washed, and resuspended for 5 min at 37°C in CCM (trace B); or loaded with quin-2 (50 μm) for 1 hr, washed, and resuspended for 1 hr at 37°C in CFM (trace C). Each tracing is representative of at least three separate experiments.

to <20 nM and completely inhibited any effect of AlF_4^- on the cytosolic free Ca^{2+} concentration in these cells (Fig. 2).

Effects of Fluoride, GTP[γS], and GDP[βS] on the F-Actin Content in Electrically Permeabilized Cells. Electropermeabilized cells were used to further establish the specific mechanism of AlF_4^- stimulation. AlF_4^- stimulation of these cells resulted in a significant increase in the F-actin content. The maximal level (around 250% of basal; Fig. 3) was, however, lower than that observed in intact cells (Fig. 1), but the response was much more rapid (Fig. 3). To confirm that the effects of AlF_4^- on actin polymerization were mediated by G proteins, we treated permeabilized cells with GDP[βS] prior to stimulation. This stable guanine nucleotide has been shown to be a competitive inhibitor of AlF_4^--induced G-protein activation (22). The AlF_4^--induced increase in F-actin content in the electropermeabilized cells was reduced by 1 mM GDP[βS] and totally abolished by 10 mM GDP[βS] (Fig. 4*A*).

The involvement of G proteins in actin polymerization was further studied by stimulating permeabilized cells with GTP[γS] (100 μM). This stable nucleotide caused a prompt (within 1 min) increase in the F-actin content, which, after 5–10 min, reached a steady level around 250% of basal (Fig. 3). However, when only responding cells were considered, the mean increase was even higher (320 ± 21% of basal, mean ± SEM, $n = 5$). The strong phalloidin-staining in permeabilized GTP[γS]-stimulated cells exhibited a somewhat more intense fluorescence in the periphery of the cells (Fig. 5*D*). The F-actin content in intact neutrophils was not affected by GTP[γS] (data not shown), certifying that the site of action of this nucleotide is intracellular. In good agreement with the results obtained in AlF_4^--stimulated cells (Fig. 4*A*), the increase in F-actin content elicited by GTP[γS] was reduced by 1 mM GDP[βS] and totally abolished by 10 mM GDP[βS] (Fig. 4*B*).

In analogy with what is described above for AlF_4^-, the ability of GTP[γS] to induce actin polymerization was tested after reduction of the cytosolic free Ca^{2+} concentration to extremely low levels by adding 3 mM EGTA to the permeabilization medium. Reduction of the Ca^{2+} concentration to this extent has been shown to totally suppress GTP[γS]-induced phospholipase C activity in HL-60 promyelocytic cells (35). The GTP[γS]-triggered increase in F-actin content in cells suspended in this EGTA-containing medium, was largely unaffected when measured after 10 min (264% of basal, mean of two separate experiments).

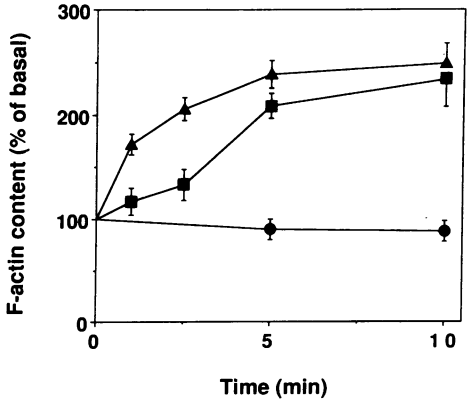

FIG. 3. Effects of AlF_4^- and GTP[γS] on the F-actin content in electropermeabilized human neutrophils. Cells (10^7 per ml) were suspended in ice-cold permeabilization medium and then subjected to repeated high-voltage discharges followed by preincubation for 2 min at 37°C. Shown are the kinetics of the response to stimulation with 20 mM NaF and 10 μM $AlCl_3$ (■; $n = 5$), 100 μM GTP[γS] (▲; $n = 8$), or buffer alone (●; $n = 4$). Results are presented as means ± SEM of n experiments.

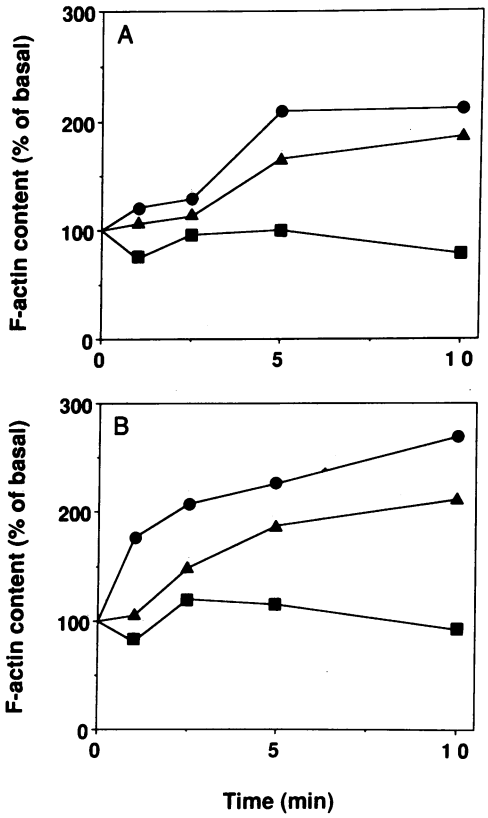

FIG. 4. Effects of GDP[βS] on the AlF_4^-- and the GTP[γS]-induced increase in F-actin content in electropermeabilized human neutrophils. Permeabilized cells (10^7 per ml) were preincubated in the permeabilization medium with 1 mM GDP[βS] (▲), 10 mM GDP[βS] (■), or no additive at all (●) for 5 min on ice, immediately followed by a 2-min preincubation at 37°C. Shown are the kinetics of the F-actin response to stimulation with 20 mM NaF and 10 μM $AlCl_3$ (*A*) or 100 μM GTP[γS] (*B*). Data are expressed as percent of their respective control—i.e., cells only pretreated with 1 mM GDP[βS], 10 mM GDP[βS], or no additive at all. Data were obtained from a single experiment and are representative of three similar separate experiments.

Electropermeabilization *per se* caused a minor increase in the F-actin content (data not shown) and a subtle swelling of the cells (Fig. 5*C*). These effects have also been observed by other investigators (36). Following the permeabilization procedure and the subsequent incubation at 37°C, it was found that ≈75% of the cells responded to GTP[γS], coinciding with the number of cells stained with trypan blue. The rest of the cells were either not permeabilized, as indicated by the fact that the majority of these nonresponding cells did not swell (Fig. 5*D*), or possibly fatally permeabilized.

DISCUSSION

G proteins play a central role in signal transduction in a number of different cell types, by regulating the activity of specific effectors, such as phospholipase C, adenylate cyclase, and ion channels in the plasma membrane (16). The fact that G proteins are able to exist in a soluble form (37–40) allows them to interact with different cytoplasmic components, for example, the cytoskeleton. This is supported by a study showing a direct interaction between G proteins and the actin filament system (41). Further, we previously observed that pertussis toxin totally inhibits chemotactic peptide-induced actin polymerization in human neutrophils (12), whereas inhibition of phospholipase C activity has no signif-

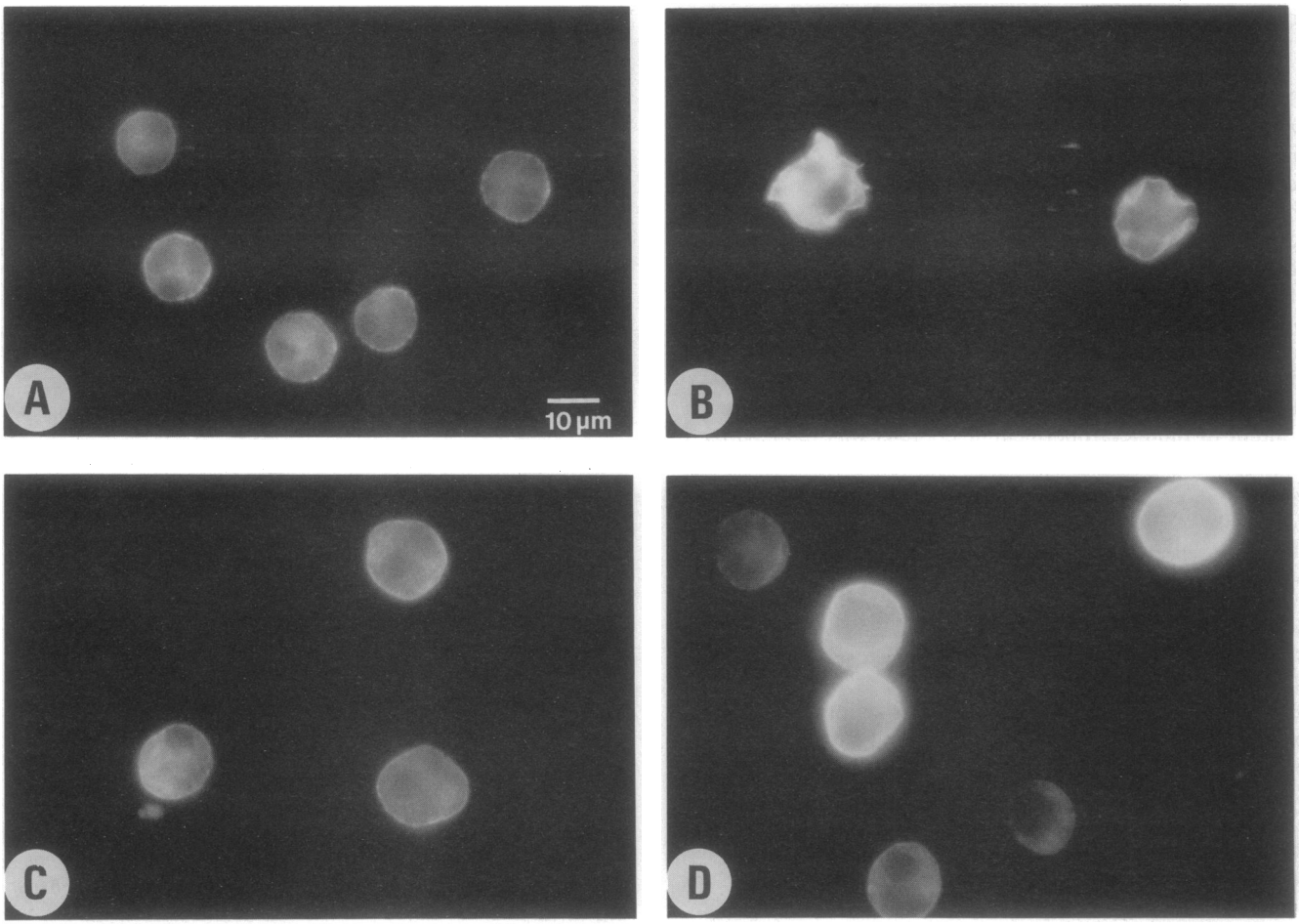

FIG. 5. Human neutrophils from different experimental groups were fixed, stained with fluorescein-phalloidin, placed between a glass microscope slide and a coverslip, and then examined and photographed in a Zeiss fluorescence microscope (Kodak Tri-X Pan 400 film developed at 800 ASA). (*A*) Intact control cells. (*B*) Intact cells stimulated with 20 mM NaF and 10 μM $AlCl_3$ for 40 min. (*C*) Electropermeabilized cells. (*D*) Electropermeabilized cells stimulated with 100 μM GTP[γS] for 10 min.

icant effect on this process (14). This indirectly suggests a regulatory role of G proteins in actin polymerization.

To pursue the idea of a close link between G proteins and actin polymerization in human neutrophils (14), the present study has taken advantage of the ability of AlF_4^- to short-cut ligand–receptor-associated events and activate G proteins in intact cells (16). This strategy excludes involvement of any signaling pathway directly associated with ligand–receptor complexes. Despite the absence of any putative signals prior to G-protein activation, AlF_4^- induced an extensive and sustained increase in F-actin content in intact human neutrophils. The AlF_4^- response was characterized by an initial lag period, similar to that preceding AlF_4^--induced activation of the respiratory burst in human neutrophils (27, 28). A reason for this lag period could be that AlF_4^- diffuses slowly to its binding site on G proteins. This is supported by the fact that AlF_4^--induced polymerization of actin was more rapid in electropermeabilized cells. AlF_4^- has been shown to affect cellular structures and systems other than G proteins (19–21). However, since GDP[βS] could antagonize the AlF_4^--induced increase in F-actin content, this response seems to have been triggered by direct activation of G proteins. Further support of a close link between G proteins and actin polymerization was provided by the findings that GTP[γS] induced a rapid actin polymerization and that this effect was inhibited by GDP[βS]. During preparation of this manuscript, similar effects of these guanine nucleotides on actin polymerization were reported by Therrien and Naccache (42). The sustained duration of both AlF_4^-- and GTP[γS]-induced increase in cellular F-actin is probably due to an inability to transform the activated G proteins to their inactive form (43).

Pretreatment with pertussis toxin totally inhibits chemotactic peptide-induced actin polymerization (12, 13). However, the same toxin only marginally affects receptor-mediated phagocytosis (10, 15), a motile event that also requires actin polymerization. One possible explanation for these findings is that human neutrophils possess pertussis toxin-sensitive G proteins coupled to chemotactic receptors, and pertussis toxin-insensitive G proteins coupled to phagocytic receptors, both involved in actin polymerization. This is supported by the present study, which shows that AlF_4^- induces an actin polymerization that is only partially reduced by pertussis toxin. Indeed, an indication that pertussis toxin-insensitive G proteins are involved in the phagocytic process comes from recent experiments revealing that GDP[βS] abolishes second-messenger generation induced by phagocytic stimuli in permeabilized human neutrophils (M. Fällman, M. Gullberg, and T.A., unpublished data). An alternative explanation that cannot be excluded is that AlF_4^- activates G proteins in a manner unaffected by ADP-ribosylation.

We previously found that neither chemotactic peptide-induced actin polymerization nor receptor-mediated phagocytosis was affected under condtions in which the activity of phospholipase C was abolished (10, 14). Under identical conditions—i.e., when the cytosolic free Ca^{2+} concentration was depressed below the level required for phospholipase C activity—neither AlF_4^-- nor GTP[γS]-initiated actin polymerization was more than marginally affected. Thus, the phos-

pholipase C-induced increase in the turnover of phosphatidylinositol 4,5-bisphosphate, or any of the number of second messengers generated by this key enzyme in the signal-transduction pathway, can be well separated from initiation of actin polymerization. Since we can, in the present study, exclude signals generated prior to the activation of G proteins, one hypothetical explanation is that actin polymerization is directly (i.e., without any intermediate links) triggered by G proteins. Indeed, several studies have suggested interactions between the subunits of G proteins and the cytoskeleton (34, 41, 44). Another interesting model for initiating actin polymerization, suggested by Lassing (45), is that the important signal is a net increase in phosphatidylinositol 4,5-bisphosphate, independent of the activity of phospholipase C. This assumption could fit with the present observations, if G proteins participate in the regulation of any of the enzymes responsible for phosphatidylinositol 4,5-bisphosphate formation. Support for such a mechanism comes from studies on membrane preparations showing that phosphatidylinositol-4-phosphate kinase is activated by GTP analogues (46, 47). This model is applicable to most of the recent studies on the phosphatidylinositol cycle and actin polymerization (1, 12, 14, 48–50).

In conclusion, based on our previous and present results, it is logical to speculate that G proteins are closely linked to the initiation of actin polymerization in human neutrophils.

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