Guanine Nucleotide–induced Polymerization of Actin in Electropermeabilized Human Neutrophils

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Abstract. The effects of exogenous guanine nucleotides on the polymerization of actin in human neutrophils were tested in an electropermeabilized cell preparation. Close to 40% permeabilization was achieved with a single electric discharge as measured by nucleic acid staining with ethidium bromide or propidium iodide with minimal (<2%) release of the cytoplasmic marker lactate dehydrogenase. In addition, electropermeabilized neutrophils retained their capacity to produce superoxide anions and to sustain a polymerization of actin in response to surface-receptor dependent stimuli such as chemotactic factors. Electropermeabilization produced a rapid and transient permeabilization that allowed the entry of guanine nucleotides into the cells. GTP and, to a larger extent, its nonhydrolyzable analog guanosine 5'-*O*-2-thiotriphosphate (GTP[S]), induced a time- and concentration-dependent polymerization of actin, as determined by increased staining with 7-nitrobenz-2-oxa-1,3-diazolylphallacidin. The effects of the aforementioned guanine nucleotides were antagonized by GDP[S], but were insensitive to pertussis toxin. Cholera toxin potentiated to a small degree the amount of actin polymerization induced by GTP[S]. These results provided direct evidence for the involvement of GTP-binding proteins in the regulation of the organization of the cytoskeleton of neutrophils, an event that is of crucial importance to the performance of the defense-oriented functions of these cells.

The accumulation of neutrophilic polymorphonuclear leukocytes (neutrophils)[1] at inflammatory sites results from the ordered and directed movement of the cells out of the circulation. The locomotory behavior of neutrophils is an essential feature of their biological functions (the body's first line of defense against foreign pathogens). Indeed, congenital defects of the contractile machinery of neutrophils leads to a severe propensity towards bacterial infections (for review see Gallin et al., 1988). The elucidation of the signaling and regulatory mechanisms controlling the movement of neutrophils, therefore, represents a central task in the comprehension of the pathophysiological basis of granulocyte functions, the first step in which is the understanding of the biochemical basis of their mechanochemical transduction apparatus (for reviews see Sha'afi and Molski, 1988; Snyderman and Verghese, 1987).

Neutrophil locomotion and phagocytosis are examples of actin-based contractile phenomena in nonmuscle cells (Stossel, 1988). Rapid and transient increases in the proportion of polymerized actin have been reported in neutrophils stimulated by chemotactic factors and other agonists (White et al., 1983; Rao and Varani, 1982; Howard and Meyer, 1984; Fechheimer and Zigmond, 1983). This cytoskeletal reorganization is thought to play an essential role in the morphological and locomotory responses of neutrophils (Sklar et al., 1985). Despite the number of studies describing the characteristics of the chemotactic factor–stimulated increase in the amount of filamentous actin in neutrophils, it has proven difficult to identify the signals that initiate this cytoskeletal reorganization, as well as the molecular identity of the cellular constituents involved. Several lines of evidence have dissociated the polymerization of actin in neutrophils from the rise of intracellular calcium that occurs essentially concurrently (Sha'afi et al., 1986; Sklar et al., 1985; Bengtsson et al., 1986). An initiating role for the cytoplasmic acidification that accompanies neutrophil stimulation has been postulated on the basis of indirect data (Yuli and Oplatka, 1987; Molski and Sha'afi, 1987; Faucher and Naccache, 1987). However, more recent experiments have shown that the acidification response could be significantly inhibited in the absence of any effect on the stimulation of the polymerization of actin (Naccache et al., 1989).

A consistent feature of the stimulated polymerization of actin in neutrophils is its sensitivity to inhibition by pertussis toxin (Shefcyk et al., 1985; Sha'afi et al., 1986; Bengtsson et al., 1986). These results have indirectly implicated a role

1. *Abbreviations used in this paper*: fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; GDP[S], guanosine 5'-*O*-2-thiodiphosphate; GTP[S], guanosine 5'-*O*-2-thiotriphosphate; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; leukotriene B_4, (5S,12R)-5,12-dihydroxy-(2,E,E,2)-6,8,10,14-eicosatetraenoic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazolylphallacidin; neutrophil, neutrophilic polymorphonuclear leukocyte.

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for guanine nucleotide–binding proteins (G proteins) in the signaling pathway(s), leading to the formation of actin filaments in neutrophils. However, pertussis toxin has been shown to affect the behavior of various cell types independently of its ADP-ribosyltransferase activity (Tamura et al., 1983; Rosoff et al., 1987; Banga et al., 1987; Gray et al., 1989) thereby stressing the need for confirmation of the conclusions drawn from pertussis toxin experiments by independent means. The purpose of the present study was to directly test for the involvement of G proteins in the initiation of actin polymerization. The strategy used was to examine the effects of the addition of guanine nucleotides to an electropermeabilized cell preparation. The results provided direct evidence for the involvement of one or more G protein(s) in the sequence of events that leads to the initiation of actin polymerization in human neutrophils.

Materials and Methods

Peripheral blood neutrophils were purified on Ficoll-Hypaque cushions after dextran sedimentation of red blood cells. The remaining erythrocytes were lysed by hypotonic shock (30 s at room temperature). The resultant suspensions of neutrophils were 97% pure and cell viability as estimated by trypan blue exclusion was >98%. Neutrophils were resuspended in calcium-free Hanks' balanced salt solution (HBSS), pH 7.4, or, when specified, in potassium buffer (110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 25 mM Hepes), pH 7.4.

Actin polymerization was measured by a modification of the 7-nitrobenz-2-oxa-1,3-diazolylphallacidin (NBD-phallacidin) staining method originally described by Howard and Oresajo (1985). When intact cells were used, 10^6 neutrophils were resuspended in 0.8 ml HBSS at 37°C. Electropermeabilization was carried out in the presence or absence of the desired nucleotides by subjecting the cell suspensions to a single electric discharge of 3.75 kV/cm^2 in 0.4 ms using a Gene Pulser (Bio-Rad Laboratories, Mississauga, Ontario, Canada). At the desired time after electropermeabilization, the cells were fixed with 3.7% formalin for 15 min at room temperature and centrifuged for 15 s at 12,000 g in a microcentrifuge. The cells were then resuspended in 0.175 ml HBSS and 25 μl of a NBD-phallacidin solution in calcium-free HBSS (8.25×10^{-7} M final concentration) containing lysophosphatidylcholine (32 μg/ml final concentration) was added. This last step resulted in a fivefold increase in the concentration of NBD-phallacidin originally used by Howard and Oresajo (1985). After a 10-min incubation at room temperature in the dark, the cells were washed free of the unbound probe by centrifugation. Cell-associated fluorescence was measured (excitation and emission wavelengths were 465 and 535 nm, respectively) in a spectrofluorimeter (SLM 8000c; SLM-Aminco, Urbana, IL). The data are expressed as ratios of the fluorescence intensities of control and treated cells. Alternatively, the degree of actin polymerization was also assessed on cells stained with NBD-phallacidin as just described on a flow cytometer (Epics C; Coulter Electronics Inc., Hialeah, FL). 2,000 cells per sample were analyzed. The shift in the population distribution of the fluorescence was taken as an index of actin polymerization (Howard and Meyer, 1984).

Superoxide production was monitored as the (superoxide dismutase-sensitive) reduction of cytochrome c by a slight modification of the method described in Metcalf et al. (1986). Briefly, neutrophil suspensions (10^6 cells/ml) were incubated under the desired experimental conditions in the presence of 120 μM cytochrome c for 5 min at 37°C. The reactions were stopped by the addition of superoxide dismutase (final concentration, 62.5 μg/ml) and the transfer of the tubes to an ice-cold bath followed by centrifugation. The optical density of the supernatants was read at 550 nm and the amount of superoxide produced was calculated using an extinction coefficient of 21.1.

The viability of permeabilized cells was assessed by the release of lactate dehydrogenase (LDH). Briefly, cells (untreated, electropermeabilized, and Triton X-100–lysed; 10^6 cells/ml) were centrifuged down and 200 μl of the supernatants were added to 1.25 ml of phosphate buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4), pH 7.35, enriched with 0.14 mg/ml $NADH^+$. The reactions were started upon the addition of 50 μl of sodium pyruvate solution and the reduction of the $NADH^+$ was monitored for 1 min by a UV-VIS spectrophotometer at 340 nm. The data are expressed as a percent of the total LDH content as determined after Triton X-100 (0.1%) lysis of the cells samples.

The permeabilization of the cells was evaluated in two ways. First, untreated, electropermeabilized, and Triton X-100–lysed cells were stained with 75 μM ethidium bromide for 20 min at room temperature. Fluorescence intensities (excitation and emission wavelengths were 365 and 580 nm, respectively) of control and permeabilized cells were compared with that of Triton X-100 (0.1%)-lysed cells in the fluorimeter. Internal controls were run to correct for the direct effects of Triton X-100 on the fluorescence of ethidium bromide. Second, untreated, permeabilized, and Triton X-100–lysed cells were stained with 80 μg/ml propidium iodide for 5 min at room temperature before being analyzed by flow cytometry using a flow cytometer (Epics C; Coulter Electronics Inc.). 5,000 cells per sample were analyzed. The extent of permeabilization was quantified by comparison of the fluorescence of electropermeabilized with Triton X-100–lysed cells.

Dextran T-500 and Ficoll-Hypaque were purchased from Pharmacia Fine Chemicals (Dorval, Québec, Canada). Lysophosphatidylcholine, sodium pyruvate solution, $NADH^+$, Triton X-100, ethidium bromide, propidium iodide, and cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO). NBD-phallacidin was from Molecular Probes Inc. (Junction City, OR). Formalin was obtained from Fisher Scientific Co. (Québec, Québec, Canada). Pertussis toxin was purchased from List Biological Co. (Campbell, CA).

Results

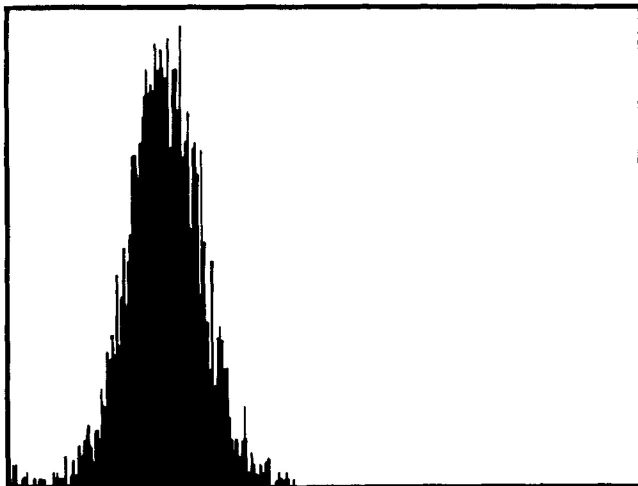
Characterization of the Electropermeabilization

The exposure of neutrophils to a single electric discharge (1,500 V, 25 μF) induced a rapid permeabilization as evidenced by the staining of nucleic acids by ethidium bromide (Table I). Since intact cells are impermeable to this dye (Gomperts, 1983), the increased fluorescence of the cell suspension after the electric discharge was an index of permeabilization. The procedure used allowed the penetration of enough dye to stain close to 40% of the nucleic acid content of the cells. Cell permeabilization was also verified by flow cytometry using propidium iodide to examine for potential nonhomogeneities in the response of the cells to the electric discharge. Control cells displayed only minimal levels of fluorescence as expected from intact cells excluding the dye. Electroporation resulted in the detection of a single peak of fluorescence that included >95% of the cells (Fig. 1), indicating that practically the entire cell population responded to the permeabilization procedure.

The effects of the electroporation on the viability of the cells was assessed by measuring the leakage of LDH. As shown in Table I, a single electric discharge increased the amount of LDH recovered in the supernatants of the cells by only ~2% in excess of that recovered from control, untreated cells. Subjecting neutrophils to two or three electric discharges increased the degree of staining with ethidium bromide; however, increasing levels of cell damage, as indicated by LDH leakage as well as progressive loss of cell responsiveness (ability to produce superoxide anions in response to formylmethionyl-leucyl-phenylalanine [fMet-Leu-Phe]), were also caused by multiple electric discharges (Table I). The degrees of permeabilization and release of lactate dehydrogenase induced by three electric discharges corresponded closely to those reported by Grinstein and Furuya (1988) using a similar procedure.

The ability of neutrophil suspensions to respond to chemotactic factors was also examined as a test of the functional viability of the electropermeabilized cells. As shown in Table I, stimulation of control and electropermeabilized cells (one discharge) with fMet-Leu-Phe or PMA resulted in the formation of equivalent amounts of superoxide. Furthermore, fMet-Leu-Phe induced similar increases in the relative amounts

A Control



B Electropermeabilized

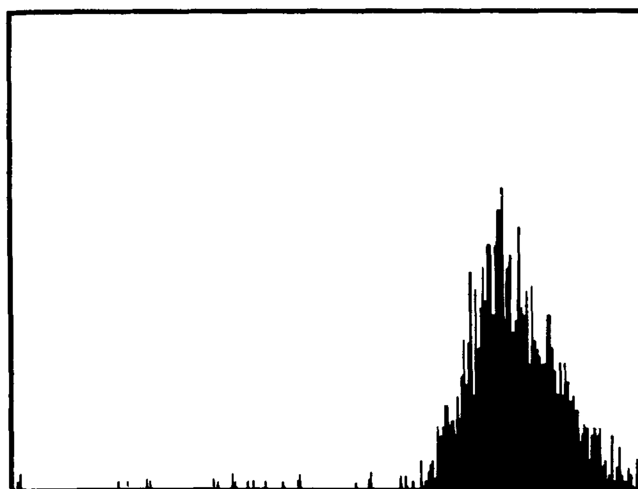


Figure 1. Characterization of the electropermeabilization by flow cytometry. In this figure, the ordinates represent the number of cells and the abscissas represent the relative fluorescence levels. The cells were treated and analyzed as described in Materials and Methods. The data shown are from a single experiment representative of three independent determinations.

of polymerized actin in control and electroporated cells (see below). These results indicated that the coupling of the chemotactic factor receptors to their effector systems (in this case, the NADPH-oxidase and the cytoskeleton) were functionally intact after electroporation (one electric discharge) and that sufficient NADPH remained in the cytoplasm to support the activation of the NADPH oxidase. Taken together, the results summarized in Table I indicated the electropermeabilization, as carried out in this study (i.e., a single electric discharge), lead to a minimal loss of macromolecules from neutrophils. On the other hand, and as previously reported (Grinstein and Furuya, 1988), cells subjected to three electric discharges did not produce any superoxide when stimulated with fMet-Leu-Phe or PMA.

Guanine Nucleotide-induced Polymerization of Actin in Electropermeabilized Neutrophils

The effects of guanosine 5′-*O*-2-thiotriphosphate (GTP[S]) on actin polymerization in control and electropermeabilized cells were investigated; the results of these studies are summarized in Table II. The responses to the chemotactic factor fMet-Leu-Phe are shown for comparative purposes. The addition of GTP[S] to nonpermeabilized cells was without effect on the level of polymerized actin in neutrophils. The level of fluorescence detected in the electropermeabilized cells diminished slightly (~20%) subsequent to the electric discharge, probably from the combination of a small loss of cells, some leakage of globular actin, and potential effects of the treatment on the actin polymerization/depolymerization equilibrium (results not shown). GTP[S] increased the amount of F-actin in electropermeabilized cells by >60%, an effect of a similar magnitude to that elicited by optimal concentrations of chemotactic factors. Furthermore, fMet-Leu-Phe increased the fluorescence ratio to about the same extent in control, untreated cells as it did in cells that had been subjected to one electric discharge.

The effects of GTP[S] on the fluorescence of NBD-phallacidin-stained neutrophils were also analyzed by flow cytometry (Fig. 2). Neither the addition of GTP[S] to nonpermeabilized cells nor the electroporation of cells in the absence of nucleotides altered the position of the peak of fluorescence. The latter was, however, shifted to the right (indicating increased fluorescence) in cells electropermeabilized in the presence of 1 mM GTP[S], a result consistent with the presence of larger amounts of polymerized actin in these cells. There were no indications of inhomogeneity in the response of the cell population to the guanine nucleotide.

The dependence of the actin polymerization response in untreated and electropermeabilized cells on the ionic composition of the suspending buffer was studied. Equivalent increases in the NBD-phallacidin fluorescence ratios caused

Table I. Characteristics of the Electropermeabilization

Conditions	EtBr staining*	LDH release‡	O_2 release§	
			fMLP	PMA
Control	0	11.4 ± 1.8	10.2 ± 1.7	17.6 ± 2.9
One discharge	39.4 ± 3.3	13.5 ± 1.7	8.30 ± 2.1	13.1 ± 6.3
Two discharges	52.3 ± 3.5	16.2 ± 2.0	ND	ND
Three discharges	86.3 ± 11.5	22.7 ± 1.8	0.14 ± 0.4	0 ± 0.9

* Ethidium Bromide (*EtBr*) staining was determined as described in Materials and Methods. Percent of the total staining achieved in cells lysed with 0.1% Triton X-100 cells. Mean ± SEM of three experiments.
‡ LDH release was determined as described in Materials and Methods. Percent of the activity recovered from cells lysed with 0.1% Triton X-100. Mean ± SEM of three experiments.
§ Superoxide release was measured as described in Materials and Methods. The concentrations of fMet-Leu-Phe (*fMLP*) and PMA were 10^{-7} and 5×10^{-8} M, respectively. Total amount of superoxide anions (in nmoles) produced by 10^6 neutrophils during a 5-min incubation at 37°C. Mean ± SEM of five experiments, each carried out in triplicate.

Table II. Effect of Guanine Nucleotides on the Level of Actin Polymerization in Human Neutrophils

Stimuli*	Electroporation‡	Actin polymerization§ (fluorescence ratios)
None	−	1.0
fMET-LEU-PHE	−	1.44 ± 0.05 (25)
GTP [S]	−	1.03 ± 0.05 (3)
None	+	1.0
fMET-LEU-PHE	+	1.29 ± 0.05 (4)
GTP [S]	+	1.64 ± 0.07 (31)

* The concentrations of fMet-Leu-Phe and GTP [S] were 10^{-7} and 10^{-3} M, respectively. The stimulation times were 30 s and 3 min, respectively.
‡ Electroporation was performed where indicated by plus signs as described in Materials and Methods (one discharge).
§ Actin polymerization was measured as described in Materials and Methods. Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate.

by GTP[S] were observed in high-sodium (HBSS) or high-potassium (K^+ buffer) solutions (1.64 ± 0.07 and 1.51 ± 0.14, respectively, mean ± SEM of three experiments carried out in triplicate).

Time Dependence of the Effect of GTP[S] on the Polymerization of Actin

The time dependence of the effect of GTP[S] on the polymerization of actin in human neutrophils was examined next (Fig. 3). In these experiments, the cells were electropermeabilized in the absence or presence of 1 mM GTP[S], and the reactions were allowed to proceed for the indicated times before being stopped by the addition of formalin. Increased polymerization of actin was detected within 20 s and reached an apparent plateau after 1 min. The stimulated level of polymerized actin was maintained for at least another 2 min. The addition of GTP[S] to nonpermeabilized cells was without effect.

The stimulation of the polymerization of actin induced by GTP[S] was dependent on the time of addition of the nucleotide relative to that of the electroporation. In these experiments, the nucleotide was added either before the electric discharge or at varying times (30, 60, and 180 s) after it. The reactions were allowed to proceed for an additional 3 min before being stopped and analyzed as described above. As shown in Fig. 4, maximal increases in fluorescence ratios were observed when GTP[S] was present in the incubation medium during the electric discharge. Progressively smaller responses were measured as the interval between the electric discharge and the addition of the nucleotide was increased. Little if any increase in fluorescence ratio was noted when GTP[S] was added 3 min after the electroporation.

Concentration Dependence of the Polymerization of Actin Induced by GTP[S]

The stimulation of the polymerization of actin induced by GTP[S] in electropermeabilized neutrophils was concentration dependent (Fig. 5). A threshold concentration of 0.1 mM GTP[S] was necessary for the detection of a significant response to the nucleotide. The magnitude of the response increased up to the highest concentration tested (1 mM).

Nucleotide Specificity of the Stimulation of Actin Polymerization

The effects of ATP, GTP, GDP, and guanosine 5'-*O*-2-thiodiphosphate (GDP[S]) on the level of polymerization of actin in electropermeabilized human neutrophils were compared with those of GTP[S] (Fig. 6). Of these nucleotides, only GTP and GTP[S] elicited significant increases in fluorescence. The response to GTP was, however, significantly smaller than that induced by an equimolar amount of its nonhydrolyzable analog GTP[S]. Furthermore, the simultaneous inclusion of equimolar amounts of GTP[S] and GDP[S] during the electroporation decreased the response to GTP[S] by ~50%. The mean actin polymerization indices were 1.64 ± 0.07 (n = 31) for GTP[S] and 1.33 ± 0.03 (n = 3) for GTP[S] plus GDP[S] ($p < 0.01$). It should be noted that the experimental protocol precluded preincubation with GDP[S].

Sensitivity of the Actin Polymerization Response of Electropermeabilized Neutrophils to Pertussis and Cholera Toxin

The effect of pertussis and cholera toxin on the ability of either fMet-Leu-Phe and (5S,12R)-5,12-dihydroxy-(2,E,E,2)-6,8,10,14-eicosatetraenoic acid (leukotriene B_4) or GTP[S] to induce actin polymerization in intact and permeabilized neutrophils, respectively, was examined next (Fig. 7). In these

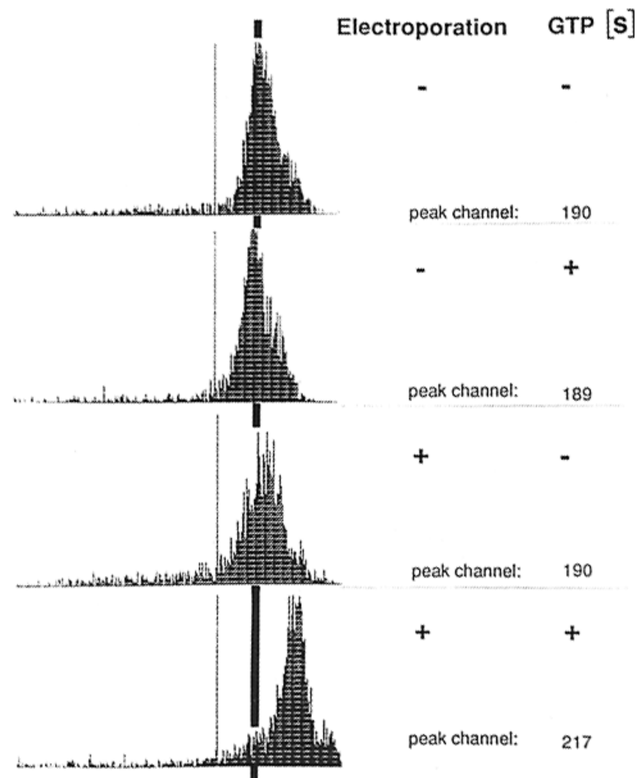


Figure 2. GTP[S]-induced actin polymerization in electropermeabilized human neutrophils as detected by flow cytometry. The cells were processed as described in Materials and Methods. The concentration of GTP[S] was 1 mM. The thick vertical bar indicates the position of the peak of fluorescence of control, untreated cells. The experimental conditions and the peak fluorescence channels are detailed to the right of the figure. The data are from a single experiment representative of three such experiments.

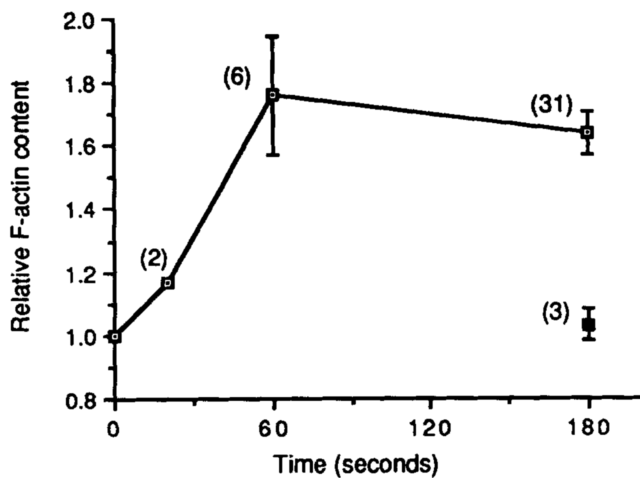


Figure 3. Kinetics of the increase in actin polymerization elicited by GTP[S] in electropermeabilized human neutrophils. GTP[S] (1 mM) was added to the cells immediately before electroporation. The experimental conditions are detailed in Materials and Methods. The open squares represent the results of the addition of GTP[S] to electropermeabilized cells. The filled square represents the effect of the addition of GTP[S] to nonpermeabilized cells. Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate (the error bar of the 30-s time points lies within the size of the symbol).

experiments, the freshly isolated cells were incubated for 3 h at 37°C with 0.5 μg/ml of either pertussis or cholera toxin before stimulation. Pertussis toxin virtually abolished the responses of intact cells to the two chemotactic factors, while cholera toxin was without any inhibitory activity. Furthermore, pertussis toxin also inhibited the polymerization of actin induced by the chemotactic factors in electropermeabilized cells (Table III). On the other hand, pertussis toxin was without any significant effect on the GTP[S]-induced polymerization of actin in permeabilized cells, while cholera toxin increased it significantly.

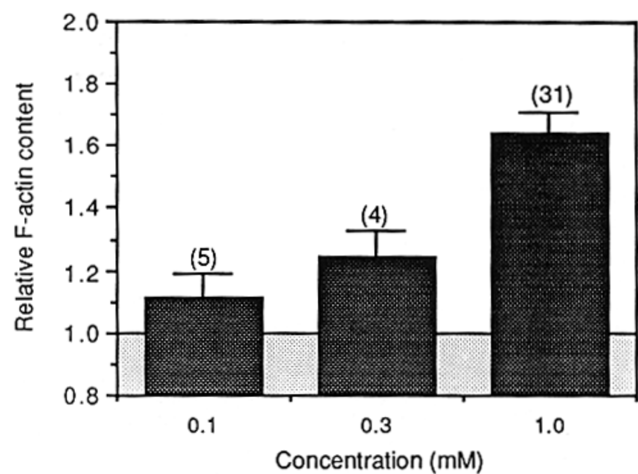


Figure 5. Concentration dependence of the stimulation of actin polymerization induced by GTP[S] in human neutrophils. The lightly shaded squares represent the results of the addition of GTP[S] to electropermeabilized cells. Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate.

Discussion

The study of the potential role of G proteins in the mediation of the effects of neutrophil agonists has relied to a great extent on the evidence derived from the use of bacterial toxins. Although it is known they specifically ADP-ribosylate different

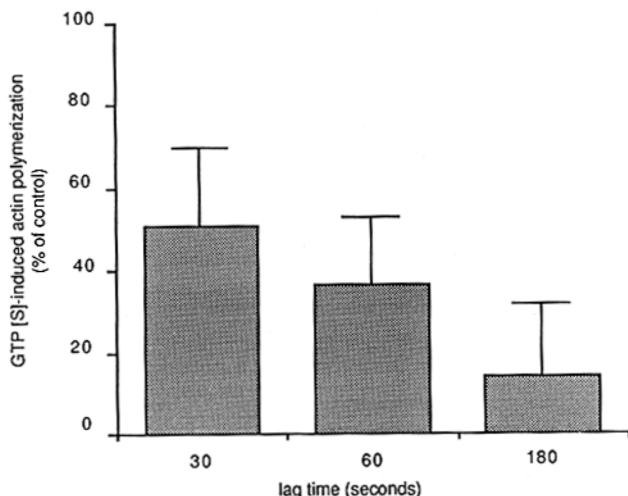


Figure 4. Effect of the delay in the addition of GTP[S] with respect to electroporation on the polymerization of actin in human neutrophils. In these experiments, GTP[S] was added either immediately before electroporation (control cells) or at the indicated times after the electric discharge. The reactions were allowed to proceed for 3 min and the cells were processed as described in Materials and Methods. The data are expressed as the percent of the increase in fluorescence ratios observed in the control cells. Mean ± SEM of four separate experiments each carried out in triplicate.

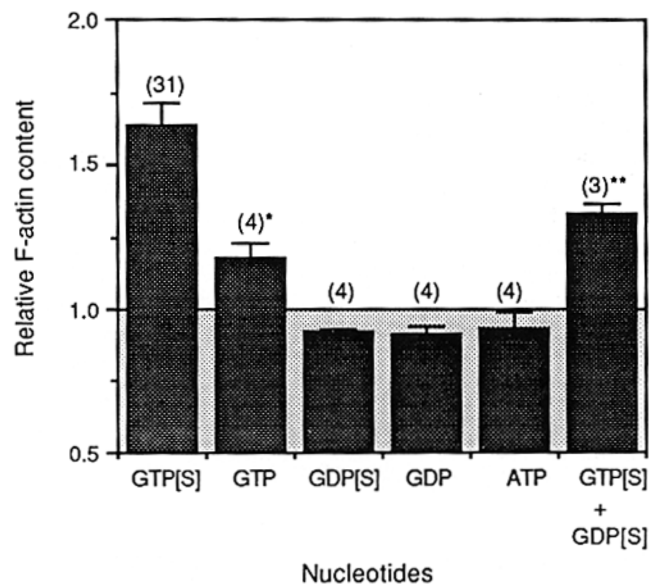


Figure 6. Nucleotide specificity of the stimulation of actin polymerization in electropermeabilized human neutrophils. The cells were electropermeabilized in the presence of 1 mM of the indicated nucleotides. Asterisks and double asterisks indicate significant differences from control ($p < 0.05$ and $p < 0.01$, respectively). Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate.

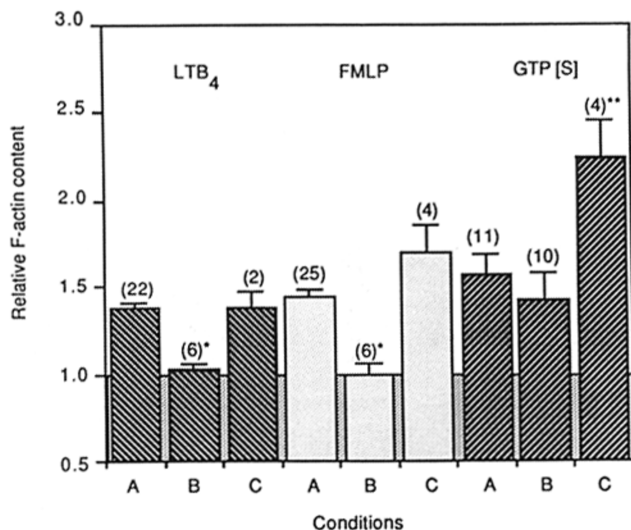


Figure 7. Effects of pertussis toxin and cholera toxin on the polymerization of actin in human neutrophils induced by chemotactic factors and GTP[S]. Intact neutrophils were stimulated with fMet-Leu-Phe (10^{-7} M) or leukotriene B_4 (10^{-7} M) for 30 or 10 s, respectively. GTP[S] (1 mM) was added during the electroporation step. Incubations with the toxins were for 3 h at 37°C with 0.5 μg/ml. (*A*) Control, untreated cells; (*B*) pertussis toxin–treated cells; (*C*) cholera toxin–treated cells. Asterisks and double asterisks indicate $p < 0.01$ and $p < 0.05$, respectively (*t* test). Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate.

subsets of these transducer elements (Gilman, 1984; Ui, 1984), recent investigations have demonstrated effects of the toxins unrelated to their ADP-ribosyltransferase activity (Tamura et al., 1983; Rosoff et al., 1987; Banga et al., 1987). Using an electropermeabilized cell preparation into which exogenous guanine nucleotides could diffuse, we have obtained data demonstrating that GTP and its nonhydrolyzable analogs directly stimulated the polymerization of actin in human neutrophils. These results have established that the activation of one or more G proteins is required for the initiation of the cytoskeletal reorganization that is an essential element of the performance of the defense functions of neutrophils.

The electroporation procedure described above yielded a viable and transiently permeabilized preparation of neutrophils into which otherwise impermeable molecules such as guanine nucleotides could penetrate. The low level of LDH leakage and the ability of the cells to sustain an oxidative burst and a polymerization of actin in response to chemotactic factors after the electroporation procedure indicated that few cytoplasmic constituents, be they small or macromolecules, were lost from the cells during the permeabilization phase and that the transduction apparatus of the cells was intact. These characteristics distinguish the present experimental conditions from those used by Grinstein and Furuya (1988) which rendered neutrophils unresponsive to chemotactic factors (superoxide production) unless exogenous NADPH was added, thereby indicating that a significant (and uncontrolled) leakage of intracellular components had taken place. The gentleness of this procedure, as well as its ease of reproducibility, are major advantages over the more commonly used detergent permeabilization protocols in studies that do not require permanent access to the cytoplasmic milieu. Indeed, the permeabilization induced by a single electric discharge was transient, lasting <3 min.

The addition of nonhydrolyzable guanine nucleotides (such as GTP[S]) to electropermeabilized neutrophils resulted in a time- and concentration-dependent increase in the amount of polymerized actin. The magnitude of this response is similar to that induced by optimal concentrations of the chemotactic factors fMet-Leu-Phe and leukotriene B_4 (White et al., 1983; Rao and Varani, 1982; Howard and Meyer, 1984; Fechheimer and Zigmond, 1983; Shefcyk et al., 1985). The time course of the effect of GTP[S] on actin polymerization is slower and more sustained than that observed in response to chemotactic factors (White et al., 1983; Rao and Varani, 1982; Howard and Meyer, 1984; Fechheimer and Zigmond, 1983; Shefcyk et al., 1985). These kinetic differences reflect the higher efficiency of the receptor-induced GDP/GTP exchange as compared with that elicited by the direct addition of the nucleotide (Gilman, 1984). GTP[S] was without effect when added to nonpermeabilized cells thereby emphasizing the requirement for access to the cytoplasmic compartment for the nucleotide to exert its effects. Though the concentrations of GTP[S] required are relatively high when compared with those necessary with isolated membrane preparations, the use of similar ranges of concentrations of guanine nucleotides (up to 1 mM) has been reported in various permeabilized cell preparations including neutrophils (Barrowman et al., 1986; Huang and Devanney, 1986; Scott and Dolphin, 1987; Knight and Baker, 1985). This need probably arises from the relatively low efficiency of incorporation of the guanine nucleotides dictated in part by the gentleness of the permeabilization procedure used in this study. The nucleotide specificity of the actin response (only GTP[S] and GTP are

Table III. Effect of Pertussis Toxin on the Polymerization of Actin Induced by Chemotactic Factors in Intact and Electropermeabilized Neutrophils

Stimuli	Treatment*	Actin polymerization (stimulation ratios)‡		
		Control	Pertussis toxin	*p*
fMet-Leu-Phe	−	1.50 ± 0.13 (3)	1.07 ± 0.07 (3)	<0.05
	+	1.45 ± 0.20 (3)	1.12 ± 0.05 (3)	<0.08
Leukotriene B_4	−	1.44 ± 0.17 (3)	1.04 ± 0.14 (3)	<0.07
	+	1.51 ± 0.06 (3)	0.98 ± 0.02 (3)	<0.01

* Treatment refers to the exposure to the electric discharge.
‡ Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in duplicate.

active in this assay) is consistent with the hypothesis that a G protein is involved. This is further supported by the inhibition of the effects of GTP[S] induced by GDP[S], a common feature of G protein-activated systems. Furthermore, the larger effects induced by GTP[S] as compared with GTP imply that the signals initiated upon the interaction of the nucleotide with its target protein are terminated upon the hydrolysis of the terminal phosphate by the GTPase inherent to the α subunit of G proteins. These various features of the actin polymerization response to GTP[S] are characteristic of G protein–mediated events. It remains to be determined whether the GTP[S]-induced polymerization of actin in permeabilized cells is related to the solubilization of actin and myosin from rabbit neutrophil membranes described by Huang and Devanney (1986).

The differential sensitivities of the effects of GTP[S] and of the chemotactic factors to the bacterial toxins raised difficult questions that are, however, not unique to this experimental system. The effects of none of the three stimuli were inhibited by cholera toxin; in fact, a potentiation of the actin polymerization response to the guanine nucleotide, the basis of which is presently unknown, was observed. On the other hand, the effects of fMet-Leu-Phe and of leukotriene B_4 in intact as well as electropermeabilized neutrophils, but not those of GTP[S], were quite sensitive to inhibition by pertussis toxin. The chemotactic factor–induced increase in the incorporation of actin in the Triton X-100–insoluble pellet of rabbit neutrophils has previously been shown to exhibit a similar sensitivity to the two toxins (Shefcyk et al., 1985). In contrast, several reports indicate that the various effects of GTP[S] are not affected by pertussis toxin. In neutrophils, Barrowman et al. (1986) observed that the potentiation of the degranulation induced by GTP[S] was not inhibited by pertussis toxin. In membranes prepared from human neutrophils (Smith et al., 1987) or differentiated HL-60 cells (Okajima et al., 1985), pertussis toxin inhibited fMet-Leu-Phe–induced augmentation of phospholipase C activity, but not the stimulation of IP_2/IP_3 formation elicited by GTP or GTP[S]. Furthermore, the time course of exocytosis in response to GTP[S] remained unaffected in pertussis toxin–treated mast cells, whereas the stimulation by compound 48/80 of the secretion was completely abolished (Lindau and Nube, 1987). These results can be explained in one of two ways.

First, several pools of G proteins may exist. These may represent distinct entities, one or more of which is insensitive to pertussis toxin although capable of transducing the signal induced by GTP[S] into an increase in the amount of polymerized actin. Alternatively, a proportion of a single G protein population may be, because of its membrane location and/or accessibility, resistant to ribosylation by the bacterial toxin. Second, GTP[S] may be able to bypass the functional inhibition induced by ribosylation of the G proteins. The examination of this hypothesis depends on the precise identification of the relevant G protein and of the effector system and the subsequent direct examination of the functional competence of the ribosylated G protein.

These various hypotheses cannot be differentiated between at present. It should be noted, however, that the insensitivity of the the effects of GTP[S] on the polymerization of actin to pertussis toxin are consistent with previous suggestions that there may be a toxin-resistant G protein in neutrophils (Barrowman et al., 1986). Whether this postulated pertussis toxin–insensitive G protein is coupled to the chemotactic factor receptors or plays other, as yet undefined, roles in neutrophil activation remains to be investigated.

In summary, the results presented in this communication indicated that GTP-binding proteins are involved in the regulatory mechanisms involved in the reorganization of the neutrophils' cytoskeleton. This conclusion is based on the finding that the direct introduction of guanine nucleotides induces, in a specific manner, a polymerization of actin in human neutrophils. Furthermore, the electropermeabilization procedure described herein should find a variety of applications since it provides a convenient means of introducing impermeable molecules into functionally responsive cells.

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References

Banga, H. S., R. K. Walker, L. K. Winberry, and S. E. Rittenhouse. 1987. Pertussis toxin can activate human platelets: comparative effects of holotoxin and its ADP-ribosylating S_1 subunit. *J. Biol. Chem.* 262:14871–14874.

Barrowman, M. M., S. Cockcroft, and B. D. Gomperts. 1986. Two roles for guanine nucleotides in the stimulus–secretion sequence of neutrophils. *Nature (Lond.).* 319:504–507.

Bengtsson, T., O. Stendhal, and T. Andersson. 1986. The role of the cytosolic free Ca^{2+} transient for fMet-Leu-Phe induced actin polymerization in human neutrophils. *Eur. J. Cell Biol.* 42:338–343.

Faucher, N., and P. H. Naccache. 1987. Relationship between pH, sodium and shape changes in chemotactic factor-stimulated human neutrophils. *J. Cell. Physiol.* 132:483–491.

Fechheimer, M., and S. Zigmond. 1983. Changes in cytoskeletal protein of polymorphonuclear leukocytes induced by chemotactic peptides. *Cell Motil.* 3:349–361.

Gallin, J. I., I. M. Goldstein, and R. Snyderman. 1988. Inflammation: Basic Principles and Clinical Correlates. Raven Press, New York. 995 pp.

Gilman, A. G. 1984. Guanine nucleotide binding regulatory proteins and dual control of adenylate cyclase. *Cell.* 36:577–579.

Gomperts, B. D. 1983. Involvement of guanine nucleotide binding protein in the gating of Ca^{2+} by receptors. *Nature (Lond.).* 306:64–66.

Gray, L. S., K. S. Huber, M. C. Gray, E. L. Hewlett, and V. H. Engelhard. 1989. Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. *J. Immunol.* 142:1631–1638.

Grinstein, S., and W. Furuya. 1988. Receptor-mediated activation of electropermeabilized neutrophils: evidence for a Ca^{2+}- and protein kinase C–independent signaling pathway. *J. Biol. Chem.* 263:1779–1783.

Howard, T. H., and W. H. Meyer. 1984. Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. *J. Cell Biol.* 98:1265–1271.

Howard, T. H., and C. O. Oresajo. 1985. A method for quantifying F-actin in chemotactic peptide activated neutrophils: study of the effect of t-BOC peptide. *Cell Motil.* 5:545–557.

Huang, C. K., and J. F. Devanney. 1986. GTP-gamma-S–induced solubilization of actin and myosin from rabbit peritoneal neutrophil membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 202:41–44.

Knight, D. E., and P. F. Baker. 1985. Guanine nucleotides and Ca-dependent exocytosis: studies on two adrenal cell preparations. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 189:345–349.

Lindau, M., and O. Nube. 1987. Pertussis toxin does not affect the time course of exocytosis in mast cells stimulated by intracellular application of GTP-gamma-S. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 222:317–321.

Metcalf, D., C. G. Begley, G. R. Johnson, N. A. Nicola, M. A. Vadas, A. F. Lopez, D. J. Williamson, G. G. Wong, S. C. Clark, and E. A. Wang. 1986. Biologic properties in vitro of a recombinant human granulocyte-macrophage colony-stimulating factor. *Blood.* 67:37–45.

Molski, T. F. P., and R. I. Shafi. 1987. Intracellular acidification, guanine-nucleotide binding proteins and cytoskeletal actin. *Cell Motil. Cytoskeleton*

8:1–6.

Naccache, P. H., S. Therrien, A. C. Caon, N. Liao, C. Gilbert, and S. R. McColl. 1989. Chemoattractant-induced cytoplasmic pH changes and cytoskeletal reorganization in human neutrophils: relationship to the stimulated calcium transients and oxidative burst. *J. Immunol.* 142:2438–2444.

Okajima, F., T. Katada, and M. Ui. 1985. Coupling of the guanine nucleotide regulatory protein to chemotactic peptide receptors in neutrophil membranes and its uncoupling by islet-activating protein, pertussis toxin: a possible role of the toxin substrate in Ca^{2+} mobilizing receptor mediated biosignalling. *J. Biol. Chem.* 260:6761–6768.

Rao, K. M. K., and J. Varani. 1982. Actin polymerization induced by chemotactic peptides and concanavalin A in rat neutrophils. *J. Immunol.* 129:1605–1608.

Rosoff, P. M., R. Walker, and L. Winberry. 1987. Pertussis toxin triggers rapid second messenger production in human T lymphocytes. *J. Immunol.* 139:2419–2423.

Scott, R. H., and A. C. Dolphin. 1987. Activation of a G protein promotes agonist responses to calcium channel ligands. *Nature (Lond.).* 330:760–762.

Sha'afi, R. I., and T. F. P. Molski. 1988. Activation of the neutrophil. *Prog. Allergy.* 42:1–64.

Sha'afi, R. I., J. Shefcyk, R. Yassin, T. F. P. Molski, M. Volpi, P. H. Naccache, J. R. White, M. B. Feinstein, and R. I. Sha'afi. 1986. Is a rise in intracellular concentration of free calcium necessary of sufficient for actin polymerization? *J. Cell Biol.* 102:1459–1463.

Shefcyk, J., R. Yassin, M. Volpi, T. F. P. Molski, P. H. Naccache, J. J. Munoz, E. L. Becker, M. B. Feinstein, and R. I. Sha'afi. 1985. Pertussis toxin but not cholera toxin inhibits the stimulated increase in actin association with the cytoskeleton in rabbit neutrophils: role of the "G proteins" in stimulus–response coupling. *Biochem. Biophys. Res. Commun.* 126:1174–1181.

Sklar, L. A., G. M. Omann, and R. G. Painter. 1985. Relationship of actin polymerization and depolymerization to light scattering in human neutrophils: dependence on receptor occupancy and intracellular Ca^{2+}. *J. Cell Biol.* 101:1161–1166.

Smith, C. D., R. J. Uhing, and R. Snyderman. 1987. Nucleotide regulatory protein–mediated activation of phospholipase C in human polymorphonuclear leukocytes is disrupted by phorbol esters. *J. Biol. Chem.* 262: 6121–6127.

Snyderman, R., and M. W. Verghese. 1987. Leukocyte activation by chemoattractant receptors: roles of a guanine nucleotide regulatory protein and polyphosphoinositide metabolism. *Rev. Infect. Dis.* 9:S562–S569.

Stossel, T. P. 1988. The mechanical response of white blood cells. *In* Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 325–343.

Tamura, M., K. Nogimori, M. Yagima, K. Ase, and M. Ui. 1983. A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. *J. Biol. Chem.* 258:6756–6761.

Ui, M. 1984. Islet-activating protein, pertussis toxin: a probe for function of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. *Trends Pharmacol. Sci.* 4:272–279.

White, J. R., P. H. Naccache, and R. I. Sha'afi. 1983. Stimulation by chemotactic factor of actin association with the cytoskeleton in rabbit neutrophils: effects of calcium and cytochalasin B. *J. Biol. Chem.* 258:14041–14047.

Yuli, I., and A. Oplatka. 1987. Cytosolic acidification as an early transduction signal of human neutrophil chemotaxis. *Science (Wash. DC).* 235:340–342.