Guanosine $5′$-$O$-$(3$-thiotriphosphate) and Guanosine $5′$-$O$-$(2$-thiodiphosphate) Activate G Proteins and Potentiate Fibroblast Growth Factor-induced DNA Synthesis in Hamster Fibroblasts

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Sonia Paris& and Jacques Pouysségur
From the Centre de Biochimie, Centre National de la Recherche Scientifique, Université de Nice, Parc Valrose, 06034 Nice Cédex, France

Addition of guanosine $5′$-$O$-$(3$-thiotriphosphate) (GTPyS) to intact Chinese hamster lung fibroblasts (CCL39) depolarized by high K$^+$ concentrations results in activation of phosphoinositide-specific phospholipase C (PLC) (at GTPyS concentrations $>0.1$ mM), inhibition of adenylate cyclase (between 10 $\mu$M and 0.5 mM), and activation of adenylate cyclase (above 0.5 mM). Since GTPyS-induced activation of PLC is dramatically enhanced upon receptor-mediated stimulation of PLC by $\alpha$-thrombin, we conclude that in depolarized CCL39 cells GTPyS directly activates various guanine nucleotide-binding regulatory proteins (G proteins) coupled to PLC ($G_\text{c}$) and to adenylate cyclase ($G_\text{i}$ and $G_\text{s}$).

Pretreatment of cells with pertussis toxin strongly inhibits GTPyS-induced activation of PLC and inhibition of adenylate cyclase. GTPyS cannot be replaced by other nucleotides, except by guanosine $5′$-$O$-$(2$-thiodiphosphate) (GDP$\beta$S), which mimics after a lag period of 15–20 min all the effects of GTPyS, with the same concentration dependence and the same sensitivity to pertussis toxin. We suggest that GDP$\beta$S is converted in cells into GTP$\beta$S, which acts as GTPyS.

Since cell viability is not affected by a transient depolarization, these observations provide a simple method to examine long-term effects of G protein activation on DNA synthesis. We show that a transient exposure of $G_0$-arrested CCL39 cells to GTPyS or GDP$\beta$S under depolarizing conditions is not sufficient by itself to induce a significant mitogenic response, but markedly potentiates the mitogenic action of fibroblast growth factor, a mitogen known to activate a receptor tyrosine kinase. The potentiating effect is maximal after 60 min of pretreatment with 2 mM GTPyS. GDP$\beta$S is equally efficient but only after a lag period of 15–20 min. Mitogenic effects of both guanine nucleotide analogs are suppressed by pertussis toxin.

Since the activation of G proteins by GTPyS under these conditions vanishes after a few hours, we conclude that a transient activation of G proteins facilitates the transition $G_0$ $\rightarrow$ $G_1$ in CCL39 cells, whereas tyrosine kinase-induced signals are sufficient to mediate the progression into S phase.

$G_0$-arrested cultures of the Chinese hamster lung fibroblast line CCL39 can be stimulated to reinitiate DNA synthesis by various defined mitogens, the two most potent of which are $\alpha$-thrombin and fibroblast growth factor (FGF). The observation that thrombin-induced mitogenesis is severely inhibited by pertussis toxin, a bacterial toxin known to ADP-ribosylate some G proteins (1), whereas FGF-induced mitogenesis is not, led us to propose that these two growth factors use different transduction pathways (2). Similar results have been reported in other cell systems. Pertussis toxin selectively inhibits the mitogenic actions of bombesin in Swiss 3T3 cells (3), serotonin in bovine aortic smooth muscle cells (5), and in CCL39 cells (6), thrombin in BCSH muscle cells (7), and lysophosphatidate in fibroblasts (8), whereas it does not affect the stimulation of DNA synthesis by platelet-derived growth factor (3–5), epidermal growth factor (EGF) (4, 8), or FGF (7). Thus, mitogens can be classified into two groups which strongly synergize with each other: the first one absolutely requires the activation of a pertussis toxin-sensitive G protein for mitogenicity whereas the second one, composed of peptide growth factors known to activate receptor tyrosine kinases (9, 10), does not. All these studies however do not firmly establish that mitogens of the first class act exclusively through G proteins. For most of them, the receptor has not yet been characterized and it is even not certain whether mitogenesis involves a single receptor. One possible approach to determine whether the activation of G-protein pathways is not only necessary but also sufficient for this family of growth factors is to attempt to mimic their action by direct receptor-independent activation of G proteins. Fluoroaluminate AlF$^+$ can be used to activate G proteins in intact CCL39 cells (11, 12) and we have shown that it can induce the same early cellular responses as thrombin (13) but its toxicity precludes long-term studies. GTPyS is known to also cause activation of G proteins in the absence of hormones but the use of this analog has been restricted to cell-free preparations or permeabilized cells (see for reviews Refs. 14–17), because it is widely accepted that it is unable to penetrate the plasma membrane and G proteins are believed to be located at the cytoplasmic side of the plasma membrane (18). Although some effects of GTPyS on G protein-mediated processes in intact cells have

1 The abbreviations used are: FGF, fibroblast growth factor; EGF, epidermal growth factor; GTPyS, guanosine $5′$-$O$-$(3$-thiotriphosphate); GDP$\beta$S, guanosine $5′$-$O$-$(2$-thiodiphosphate); GTP$\beta$S, guanosine $5′$-$O$-$(2$-thiodiphosphate); G protein, guanine nucleotide-binding regulatory protein; $G_\text{s}$, stimulatory G protein of adenylate cyclase; $G_\text{i}$, inhibitory G protein of adenylate cyclase; $G_\text{c}$, stimulatory G protein of phospholipase C, IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco’s modified Eagle’s medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPs, total inositol phosphates; PLC, phosphoinositide-specific phospholipase C.
been occasionally observed (19–21), they have been attributed either to the activation of surface receptors (20–21) or to some nonspecific effects unrelated to G proteins (19).

Here we report that high concentrations of GTPγS are indeed able to cause a substantial activation of various G proteins (Gp(s) coupled to PLC, Gi, and Gs coupled to adenylate cyclase) in intact CCL39 cells, provided that the cells are depolarized by incubation in a high-K⁺ medium. Unexpectedly, GDPβS can likewise activate G proteins after a prolonged incubation. Taking advantage of these observations, we provide a simple method to examine long-term effects of G protein activation in intact cells, which show that a transient activation of G proteins by GTPγS or GDPβS is sufficient to potentiate FGF’s mitogenic effects to a similar extent as low concentrations of thrombin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant basic FGF was a gift of Dr. D. Gospodarowics (University of California Medical Center, San Francisco, CA). Highly purified human α-thrombin (2860 NIH units/ml) was generously provided by Dr. J. W. Fenton II (New York State Department of Health, Albany, NY). Crystalline bovine insulin was from Sigma. GTPγS and GDPβS were from Boehringer Mannheim, Nigericin, forskolin, and IBMX were from Sigma. Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). myo-[α-3H]-inositol, [α-3H]adenine, and [methyl-3H]thymidine were from Amersham Corp.

**Cell Culture**—The Chinese hamster fibroblast-like CCL39 (American Type Culture Collection) was grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 5% (v/v) fetal calf serum, antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) and 25 mM bicarbonate at 37 °C in 5% CO2, 95% air. Confluent cultures in 35-mm dishes (~0.3 mg of protein) were labeled to equilibrium with [3H]inositol (1 μCi/ml) over the 24-h incubation in serum-free DMEM required for Gα arrest. Cells were then washed with a Hepes-buffered saline solution containing either 130 mM NaCl (135 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM Hepes/Tris pH 7.4) or 135 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM Hepes/Tris pH 7.3) and were equilibrated for 15 min at 37 °C in the same medium, supplemented in the case of the 135 mM K⁺ saline with 1 μM nigericin. The medium was then removed and replaced with 0.7 ml of the same solution supplemented with 10 mM LiCl, and after 5 min the formation of [γ-3H]inositol phosphates was initiated by adenosyl-cyclase or by the addition of 1 mM GTPrS, as indicated. Results were determined at desired times by addition of 0.1 ml of 35% (w/v) HClO₄ and immediate incubation on ice. The acid extract was removed and the dish rinsed with 0.5 ml of H₂O. The combined extract and wash was neutralized with KOH and centrifuged to eliminate the KClO₄ precipitate. The neutralized extract was loaded onto anion-exchange columns (Bio-Rad AG 1-XA, formate form) (22). Free inositol, glycerophosphoinositol and total inositol phosphates (IPs) were successively eluted with (a) 5 ml of 5 mM, (b) 5 ml of 40 mM, and (c) 5 ml of 2 M ammonium formate/formic acid, pH 5, respectively. When the duration of GTPγS-stimulated phospholipase C activity was examined, the cultures were treated as described in the legend to Fig. 1, and IP accumulation was induced by adding 20 mM LiCl at the indicated times. The reaction was terminated by aspirating the medium and adding 0.5 ml of ice-cold 7% (w/v) HClO₄. The acid extract was removed and the dish rinsed with 0.5 ml of H₂O.

The combined extract and wash was then treated as above.

**Measurement of Cyclic AMP Production**—Adenylate cyclase activity was measured in intact cells as the conversion of [γ-3H]ATP to [α-3H]cAMP after cellular incorporation of [α-3H]adenine, as described previously (12). Briefly, confluent cultures in 35-mm dishes were labeled with [α-3H]adenine (2 μCi/ml) over the 24-h incubation in serum-free DMEM. Cells were then washed with the 135 mM K⁺-containing medium before adding 1 mM GTPrS or GDPβS (Li⁺ salts) as detailed for each experiment. The pretreatment was terminated by quickly aspirating the medium and washing the cultures with Hepes-buffered DMEM, pH 7.4, at 37 °C.

**RESULTS**

**High Concentrations of GTPγS Stimulate Phosphoinositide Breakdown in Intact CCL39 Cells**—Fig. 1 (left) shows that addition of 1 mM GTPγS to intact CCL39 cells in a physiological salt solution containing 130 mM Na⁺ induces a small but significant activation of a phosphoinositide-specific phospholipase C activity (PLC) as evidenced by the accumulation of inositol phosphates in the presence of Li⁺. This activation, however, is markedly enhanced when external Na⁺ is entirely replaced with K⁺ (Fig. 1, right). It should be noted that in the latter case cells were equilibrated for 15 min in high-K⁺ medium in the presence of 1 μg/ml nigericin before GTPγS addition. Under these conditions no basal PLC activity can be detected with Li⁺ alone, whereas in absence of nigericin replacement of Na⁺ with K⁺ causes a transient spontaneous activation of IP formation, which is due at least in part to the intracellular acidification resulting from Na⁺ removal.² By

² S. Paris, manuscript in preparation.
acting as a K*/H* exchanger across the plasma membrane, nigericin causes equilibration of pH* with pH, when K* ~ K* (23) and accelerates the collapse of this basal PLC activity, thus facilitating the study of GTPγS effects.

Iso-osmotic substitution of Na+ by N-methyl-D-glucamine does not result in an enhancement of GTPγS-induced IP formation (not shown), which indicates that the effects of high-K* medium are not due to removal of Na* but most likely to cell depolarization.

As shown in Fig. 2, high concentrations of GTPγS are required to activate PLC in high-K* medium. The activation becomes detectable only above 0.1 mM and 3 mM is not sufficient to give maximal stimulation.

In these experiments, the incubation medium was saved for IP determination, since it was observed that the efflux of free [3H]inositol was increased in K* medium as compared with Na* medium, which first suggested to us that depolarization might have rendered cells permeable to small molecules, possibly including IPs. But in fact the radioactivity recovered in the extracellular medium as IPs was very low, and quite similar results were obtained when measuring total (in + out) or only intracellular IPs (not shown).

GDPβS Mimics the Effect of GTPγS after a Lag Period and Both Stimulatory Effects Are Inhibited by Pertussis Toxin—Surprisingly, 1 mM GDPSβ also causes IP formation in depolarized CCL39 cells, but with a marked latency as compared with the effect of GTPγS (Fig. 3). Whereas IPs accumulate without any appreciable lag in GTPγS-stimulated cells (Fig. 3, left), an incubation of at least 15–20 min with GDPSβ is required before the cellular IP content elevates above basal (Fig. 3, right). Thereafter, however, the rate of IP formation progressively increases in GDPβS-stimulated cells and consequently the difference in effectiveness between the two nucleotides tends to dissipate after long incubations. Table I shows that a 1-h exposure to 1 mM GTPγS or GDPβS in high K* medium followed by washout of the nucleotides and return to a physiological culture medium results in a very similar PLC activity in both cases.

Pretreatment of cells with 100 ng/ml pertussis toxin causes a marked attenuation of both GTPγS- and GDPβS-induced formation of IPs (Fig. 3). This concentration of pertussis toxin was routinely used since it had been observed to give maximal inhibition in CCL39 cells of both thrombin- and AlF* induced PLC activities (24, 11), but in fact Fig. 4 shows that increasing the toxin concentration up to 1 μg/ml causes a further inhibition of GTPγS-stimulated IP release. Thus, the dose-response curve for pertussis toxin inhibition appears to be biphasic, which suggests the presence in these cells of two PLC-coupled G proteins with a differential sensitivity to pertussis toxin.

Thrombin Strongly Potentiates GTPγS-induced IP Release—As shown in Fig. 5, addition of 1 unit/ml thrombin to quiescent CCl39 cells incubated in high-K* medium induces

![Figure 2](image-url)  
**Fig. 2.** Concentration dependence of GTPγS-induced IP formation in high-K* medium. Conditions were as described for Fig. 1 (right) except that the concentration of GTPγS was varied from 0 to 3 mM and the intracellular Li* concentration adjusted in all cases to 20 mM. Total IPs were determined after 30 min.

![Figure 3](image-url)  
**Fig. 3.** Compared time courses of GTPγS- and GDPβS-induced IP formation in high-K* medium: effect of pertussis toxin. Conditions were as described for Fig. 1 (right). At time 0 the cultures, pretreated (O) or not (O) with 100 ng/ml pertussis toxin for 6 h, received 1 mM GTPγS (left) or 1 mM GDPβS (right). Control dishes were incubated with Li* alone (□).

![Figure 4](image-url)  
**Fig. 4.** Dose-response curve for the inhibition by pertussis toxin of GTPγS-induced IP formation. Cells were pretreated with varying concentrations of pertussis toxin for 8 h before being incubated in high-K* medium and receiving Li* and 1 mM GTPγS as described for Fig. 1 (right). The accumulation of inositol phosphates was measured after 45 min. In the absence of GTPγS the IP content of cells treated with Li* for 45 min was not affected by pertussis toxin pretreatment. This basal value (~1,600 dpm) was subtracted. Thus 100% refers to the GTPγS-induced IP formation measured in control cells not treated with the toxin. Values are means of duplicate determinations which differed by less than the size of symbols.
a rapid but transient IP formation. Since 10 mM Li+ is not sufficient for complete inhibition of inositol monophosphate phosphatase at high inositol monophosphate concentrations (25), the cellular IP content appears to decline even between 5 and 30 min after thrombin addition, indicating that the rate of inositol monophosphate degradation becomes higher than the rate of IP formation. This rapid decline of thrombin-induced PLC activity is certainly due for the most part to the homologous desensitization to thrombin that we have previously observed (26), but in addition the absence of Na+ in the medium seems to greatly impair the coupling of thrombin receptors with G proteins.3 Addition of 1 mM GTP\(_\gamma\)S to thrombin prevented the decay of thrombin's stimulation, consistent with the interpretation that activated thrombin receptors accelerate guanine nucleotide exchange on G proteins and thus facilitate GTP\(_\gamma\)S binding, leading to a strong and sustained activation of PLC. Pertussis toxin pretreatment similarly reduces IP accumulations induced by GTP\(_\gamma\)S, thrombin or GTP\(_\gamma\)S plus thrombin (Fig. 5, inset).

GTP\(_\gamma\)S and GDP\(_\beta\)S Excite a Dual Effect on Stimulated Adenylate Cyclase—Fig. 6 shows the effects of a 60-min pretreatment of CCL39 cells with increasing concentrations of GTP\(_\gamma\)S or GDP\(_\beta\)S on forskolin-stimulated adenylate cyclase. Both nucleotides exert under these conditions a similar dual effect on stimulated adenylate cyclase, with a dose-dependent inhibition of cAMP formation up to 0.5 mM and a progressive relief of the inhibition above this concentration. Such a biphasic dose response has been observed for thrombin effects on cAMP formation in CCL39 cells and we have demonstrated that in that case, it is due to the superposition of a G\(_i\)-mediated inhibition with a protein kinase C-mediated activation (12). In the case of GTP\(_\gamma\)S the situation appears even more complex since high concentrations of GTP\(_\gamma\)S also increase the basal cyclase activity, in the absence of forskolin, with a 2-fold stimulation at 3 mM GTP\(_\gamma\)S (not shown). This effect cannot be ascribed to protein kinase C since only stimulated adenylate cyclase is affected by protein kinase C activators (12). Therefore, a direct activation of G\(_i\) is likely to underlie, possibly together with protein kinase C activation, the suppression of adenylate cyclase inhibition observed at high GTP\(_\gamma\)S concentrations.

Pertussis toxin pretreatment completely abolishes the inhibitory effects of both GTP\(_\gamma\)S and GDP\(_\beta\)S on adenylate cyclase, which demonstrates that these effects are due, as for thrombin, to activation of G\(_i\).

Interestingly also, depolarization of cells does not seem to dramatically affect their permeability to cAMP since measurement of total (intra- plus extracellular) cAMP as in Fig. 6 or of only intracellular cAMP (not shown) led to very similar results. Less than 20% of cAMP was found in extracellular medium after a 5-min stimulation with forskolin and IBMX in high-K+ medium.

Cell Permeability to Phosphorylated Compounds Is Not Increased in High-K+ Medium—Although the results presented above already demonstrated that depolarization by high-K+ does not cause a marked efflux of inositol phosphates or cAMP, the possibility of an increased permeability to phosphorylated compounds, which could explain the entry of GTP\(_\gamma\)S into depolarized cells, was further examined with [\(^3\)H]2-deoxyglucose. The glucose analog 2-deoxyglucose enters CCL39 fibroblasts via the d-glucose facilitated transport system and accumulates in cells mainly as 2-deoxyglucose 6-phosphate (27). Measurement of the efflux of the latter compound has already been used as a test for membrane permeability (28). Therefore CCL39 cells were incubated for 2.5 h with the labeled sugar in glucose-free DMEM and efflux of the intracellular radioactivity was then compared in Na+ and K+ media. No significant difference was observed between the two conditions. In both media, there was a fast exit in the first minutes of a small fraction (~20%) of the initial radioactivity, presumably corresponding to the free [\(^3\)H]2-deoxyglucose present in cells, but no further significant efflux occurred over the next 30 min (data not shown).

A Transient Exposure to GTP\(_\gamma\)S in High-K+ Medium Results in a Marked Potentiation of FGF-induced DNA Synthesis—In the experiment depicted in Fig. 7, Go-arrested CCL39 cells were exposed for 30 min in high-K+ medium containing 1 \(\mu\)g/ml nigericin and the indicated concentrations of GTP\(_\gamma\)S in an isotonic saline containing either NaCl (Fig. 7, right) or KCl (Fig. 7, left) as the main salt. Cells were then

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3 S. Paris, unpublished results.

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**Fig. 5.** Synergistic effects of GTP\(_\gamma\)S and thrombin on IP formation in high-K+ medium. Conditions are as described for Fig. 1 (right). At time 0 (5 min after Li+), the cultures were stimulated with 1 mM GTP\(_\gamma\)S (C), 1 unit/ml thrombin (O), or both (3). Unstimulated cells were incubated with Li+ alone (A). Total IPs were measured at the indicated times. Inset, the IP accumulation after 50 min in control cells (hatched bars) or in cells pretreated for 6 h with 100 ng/ml pertussis toxin (PT) (dark bars) after stimulation with 1 mM GTP\(_\gamma\)S, 1 unit/ml thrombin (THR), or both. Basal value (not affected by the toxin treatment) was subtracted.

**Fig. 6.** Compared effects of GTP\(_\gamma\)S and GDP\(_\beta\)S (after 60 min of pretreatment) on forskolin-stimulated cAMP production. Effect of pertussis toxin. [\(^3\)H]Adenine-labeled cultures were pretreated for 6 h with (3) or without (O) 100 ng/ml pertussis toxin (PT). Cells were then incubated for 1 h in 0.7 ml of high-K+ medium containing 1 \(\mu\)g/ml nigericin and the indicated concentrations of GTP\(_\gamma\)S (left) or GDP\(_\beta\)S (right). IBMX (1 mM) was added for the last 5 min of this preincubation. The culture was then stimulated with 50 \(\mu\)M forskolin and the reaction was stopped after 5 min by addition of 0.3 ml of 15% trichloroacetic acid, for determination of total (intra- and extracellular) [\(^3\)H]ATP and [\(^3\)H]cAMP as described under "Experimental Procedures."
GTPrS and GDPβS Potentiate FGF-induced DNA Synthesis

Fig. 1. Effect of a 30-min pretreatment with GTPrS in an isotonic KCl versus NaCl salt solution on the stimulation of DNA synthesis by various growth factors. G0-arrested cultures were exposed for 30 min to the indicated concentrations of GTPrS in an isotonic salt solution containing 135 mM KCl (left) or 130 mM NaCl (right) as described under "Experimental Procedures." GTPrS was then washed away and incubations were continued for 18 h in 1:1 DMEM/Ham's F-12 medium containing [3H]thymidine and the indicated growth factors: 25 ng/ml FGF (C), 1 unit/ml thrombin (THR) (O), 10 μg/ml insulin (INS) (△), or no growth factor (○). The incorporation of [3H]thymidine into DNA was measured as described under "Experimental Procedures."

FIG. 7. Effect of a 30-min pretreatment with GTPrS in an isotonic KCl versus NaCl salt solution on the stimulation of DNA synthesis by various growth factors. G0-arrested cultures were exposed for 30 min to the indicated concentrations of GTPrS in an isotonic salt solution containing 135 mM KCl (left) or 130 mM NaCl (right) as described under "Experimental Procedures." GTPrS was then washed away and incubations were continued for 18 h in 1:1 DMEM/Ham's F-12 medium containing [3H]thymidine and the indicated growth factors: 25 ng/ml FGF (C), 1 unit/ml thrombin (THR) (O), 10 μg/ml insulin (INS) (△), or no growth factor (○). The incorporation of [3H]thymidine into DNA was measured as described under "Experimental Procedures."

washed free of extracellular GTPrS and transferred into a serum-free culture medium supplemented or not with various growth factors. Reinitiation of DNA synthesis was monitored by measurement of the cumulative incorporation of [3H] thymidine over 18 h. This incubation time was chosen because it corresponds to the peak of S-phase reentry for FGF-stimulated cells. In contrast to thrombin, which gives a sustained mitogenic stimulation and allows continuous entry of cells into S-phase from 12 h to 30 h after its addition (29), FGF was found to cause only a transient stimulation of G0-arrested CCL39 cells, with a rapid decline of the number of cells in S-phase (as measured by pulse labeling with [3H]thymidine) after 18 h. This is presumably due to trapping of FGF into the extracellular matrix (30), since FGF "deactivation" is more dramatic in confluent cultures than in sparse cells and it can be partly overcome by increasing FGF concentration or adding heparin, known to compete with the extracellular matrix for binding FGF (30). Thus, if thrombin has seemed to be a better mitogen for CCL39 cells than FGF after 24 h (31, 32) it is essentially because of the persistence of thrombin's stimulatory effects. But, in fact, after 18 h (Fig. 7) the same mitogenic response was obtained with 25 ng/ml FGF or 1 unit/ml thrombin, concentrations which give 80% of the maximal response for both mitogens.

As shown in Fig. 7 (right), a pretreatment of CCL39 cells with GTPrS in an isotonic NaCl solution did not greatly affect the subsequent mitogenic response to various growth factors, since the mitogenic activities of FGF and thrombin were both maximally increased by ~30%. In contrast, when GTPrS pretreatment was performed in a high-K" medium (Fig. 7, left), a strong synergistic interaction occurred between GTPrS and FGF. Whereas pretreatment with GTPrS alone was not sufficient to elicit a significant DNA synthesis (it caused only a doubling of the basal [3H]thymidine incorporation), it resulted in a large dose-dependent potentiation of FGF's mitogenicity, with a 3.6-fold stimulation at 2 mM GTPrS. This synergistic combination of GTPrS and FGF was as effective in stimulating thymidine incorporation as the combination of 25 ng/ml FGF and 10^{-7} unit/ml thrombin (see Fig. 10), which yields ~70% of the maximal mitogenic response that can be obtained in CCL39 cells.

Most interestingly, GTPrS pretreatment failed to significantly enhance the mitogenic response to thrombin, in marked contrast with FGF (thrombin-induced thymidine incorporation was increased by only ~20%). Some synergism was observed between insulin and GTPrS, with a 2.5-fold amplification of insulin effect at maximal GTPrS concentrations, but as insulin by itself is a very weak mitogen for CCL39 cells, the stimulation by GTPrS was not sufficient to elicit a substantial mitogenic response.

Replacement of Na" with N-methyl-D-glucamine in the saline solution used for GTPrS pretreatment did not mimic the effects of high K" (not shown) which indicates that K" effects are not due to Na" removal but most likely to depolarization of cells.

GDPβS Mimics the Mitogenic Effects of GTPrS But With a Lag period—As shown in Fig. 8, a transient exposure (45 min) to GTPrS in high-K" saline affected the mitogenic effects of thrombin and FGF in a very similar way as GTPrS pretreatment. While the response to thrombin was only slightly increased, the mitogenic effect of FGF was greatly enhanced with a dose-response for GDPβS very comparable to that observed with GTPrS (Fig. 7): with both nucleotides maximal effect was obtained at 2 mM and half-maximal effect at 0.5 mM. Comparison of the potentiating effects of a same concentration of GDPβS and GTPrS (Fig. 8) shows, however, that GTPrS remained slightly more potent than GDPβS under these conditions.

The influence of the time of exposure to GTPrS and GDPβS in high-K" medium was next examined in Fig. 9. With GTPrS, a time of pretreatment as short as 5 min was sufficient to cause a significant enhancement of FGF-induced DNA synthesis, but the amplitude of the potentiation increased with the duration of GTPrS exposure, and leveled off after 40-60 min. In contrast, a minimal exposure of 30 min to GDPβS was required to elevate FGF's mitogenicity above the control value and thereafter GDPβS-induced stimulation increased steadily with time of pretreatment up to 1 h, the last time point measured. A similar pattern emerged with insulin, even though the magnitude of the response was lower. The stimulatory effect of guanine nucleotide on insulin-induced [3H]thymidine incorporation was detectable after 30 min for GTPrS but only after 45 min for GDPβS. Thus, with both mitogens, GDPβS mimics the effects of GTPrS but only after a lag phase of 15-20 min. In contrast, none of the other nucleotides tested, including GTP, GDP, ATP, and ADP,
A Pertussis Toxin-sensitive G Protein Is Involved in GTPγS-mediated Stimulation of DNA Synthesis—Data presented in Fig. 10 first confirm our previous observations (2) that pertussis toxin has no effect on FGF-induced mitogenesis, whereas it strongly inhibits the mitogenic response to thrombin. Furthermore, Fig. 10 (left) shows that the synergistic effects of GTPγS on FGF-induced thymidine incorporation were progressively suppressed with increasing concentrations of pertussis toxin. However, the dose-response curve for pertussis toxin inhibition appeared markedly shifted to the right as compared to that obtained in thrombin-stimulated cells. Indeed, whereas 1 ng/ml pertussis toxin was sufficient to cause maximal inhibition of thrombin’s mitogenicity, this concentration had no significant effect on the rate of accumulation of inositol phosphates. Results presented in Fig. 10 clearly demonstrate, as mentioned above, that a pretreatment with 1 mM GTPγS is exactly equivalent, both in mitogenicity and in sensitivity to pertussis toxin, to 10⁻³ unit/ml thrombin, a concentration which by itself is not mitogenic (33) but strongly synergizes with FGF (34).

High concentrations of pertussis toxin likewise suppressed the synergetic effects of GDPβS on FGF-induced mitogenesis (data not shown).

Since both GTPγS and thrombin appeared to involve pertussis toxin sensitive G protein(s) in their mitogenic actions, it was of interest to determine whether they both activate the same G protein(s). To address this question, we took advantage of the observation made in CCL39 cells as in other cell systems (35) that pertussis toxin is still able to inhibit thrombin’s mitogenicity when it is added at the same time as thrombin, instead of several hours before, even though the dose-response is then slightly shifted toward high concentrations (compare Fig. 10, right, and Fig. 11). Moreover, since pertussis toxin is known to ADP-ribosylate only the heterotrimeric form of G proteins (1), we expected that prior activation by GTPγS would cause dissociation of G proteins and make them refractory to ADP-ribosylation by pertussis toxin, as demonstrated for G₁ in mast cells (36). Therefore, CCL39 cells were first transiently exposed to GTPγS in high-K⁺ medium and then stimulated by thrombin in the presence of varying concentrations of the toxin. Consequently, the sensitivity of thrombin’s mitogenicity to pertussis toxin was markedly attenuated, as shown in Fig. 11, with a further shift to the right of the dose-response curve by approximately 1 order of magnitude, but it was clearly not completely abolished. This result therefore suggests that 1) GTPγS did activate the pertussis toxin-sensitive G protein(s) involved in thrombin’s mitogenic pathway, but 2) this activation was either too low or too transient to completely protect the G protein(s) from ADP-ribosylation throughout the entire progression of cells through the G₁ phase of the cell cycle.

GTPγS Pretreatment Causes Only a Transient Activation of Phospholipase C-coupled G Protein(s)—To assess the duration of GTPγS effects on G proteins after washout of extracellular GTPγS, we chose to measure G₁, mediated activation of inositol lipid phospholipase C, since the Li⁺ assay provides a simple and sensitive method. Therefore GTPγS-pretreated cells were pulsed with Li⁺ at various times after their transfer into GTPγS-free culture medium, supplemented or not with FGF, and PLC activity was determined by the rate of accumulation of inositol phosphates. Results are presented in Fig. 12 and are compared with the duration of thrombin-induced PLC activity. Although 1 mM GTPγS
in high-K⁺ medium is by far less efficient than 1 unit/ml thrombin for stimulating PLC during the first few minutes of PLC to thrombin (26) that the enzyme activity was much higher in GTPyS-treated cells than in thrombin-stimulated cells after 1 h. However, whereas the rate of IP formation remained remarkably stable for at least 7 h in thrombin-desensitized cells, as described previously (25), it rapidly declined in GTPyS-treated cells within a few hours following treatment. When FGF was added, in order to mimic the conditions under which mitogenic effects of GTPyS are best revealed, PLC activity was found to be enhanced by ~50% as compared with GTPyS alone. This increase, which substantiates our earlier observations that FGF potentiates G₁₅-mediated activation of PLC (34), was maintained throughout the duration of the experiment, but it was insufficient to prevent PLC activity from dropping below thrombin-induced level after 3 h and becoming hardly detectable after 5 h. These results therefore clearly demonstrate that, even in the presence of FGF, the activation of G₁₅(s) by GTPyS remains elevated for only a few hours after removal of extracellular GTPyS and high-K⁺ medium, presumably because of the slow hydrolysis of intracellular GTPyS.

**DISCUSSION**

In this report we have shown that addition of high concentrations of GTPyS to intact depolarized CCL39 fibroblasts results in the activation of several G proteins, Gₛ, G₁₅, and G₁₃, leading to activation of PLC and inhibition or activation of adenylate cyclase, respectively. The interpretation that G₁₅(s) directly activates G proteins rather than binding to cell surface receptors coupled to G proteins is supported by the following arguments: 1) a receptor-mediated activation would not be dependent on cell depolarization; 2) activating effects of GTPyS on PLC are not mimicked by ATP, which excludes any involvement of purinergic receptors as in other cell systems (20, 21); 3) the striking synergistic interaction between GTPyS and a receptor agonist such as thrombin for PLC stimulation strongly argues in favor of a direct binding of GTPyS on the G protein(s) coupled to thrombin receptors; 4) GTPyS-induced activation of PLC persists for several hours after removal of extracellular GTPyS.

Unexpectedly, GDPβS mimics all the effects of GTPyS on Gₛ, G₁₅, and G₁₃ activities, but only after a marked lag period of about 15–20 min. The simplest interpretation is that GDPβS can be transformed by cells into GTPyS which behaves like a nonhydrolyzable analog of GTP. Indeed, GDPβS has been shown in vitro to be a substrate for nucleoside triphosphate regeneration systems such as pyruvate kinase or creatine kinase (37). It is therefore very likely that the phosphorylation of GDPβS into GTPβS can take place in intact cells. Moreover, even though it is always much less potent than GTPyS, GTPβS can activate transducin (38), bacterial elongation factor Tu (39) or ras protein (40) and it also potentiates glucagon-induced stimulation of adenylate cyclase in liver membranes, with half-maximal effect obtained at 5 µM (37).

How GTPyS and GDPβS enter depolarized cells is unclear. Depolarization does not increase cell permeability to phosphorylated compounds such as 2-deoxyglucose-6-phosphate, inositol phosphates, and cAMP. Moreover, direct measurements of [³⁵S]GTPyS uptake have shown that GTPyS does not enter CCL39 cells by passive diffusion but, very surprisingly, by a saturable process, specific for guanine nucleotides, and so whether cells are depolarized or not (data not shown). [³⁵S]GTPyS uptake is only slightly higher (by ~20%) under depolarizing conditions. Analysis of the intracellular acid-soluble radioactivity revealed, however, that [³⁵S]GTPyS and [³⁵S]GDPβS were mostly degraded to [³⁵S]thiophosphate after 1 h of incubation. This high degradation, which explains why our attempts to demonstrate the intracellular conversion of [³⁵S]GDPβS into [³⁵S]GTPβS have failed (not shown), suggests that the actual concentration of GTPyS or GDPβS really “sensed” by the G proteins is extremely low. It is likely that the modest difference observed for [³⁵S]GTPyS uptake between depolarizing and nondepolarizing conditions represents GTPyS bound to G proteins. Whether depolarization increases the concentration of GTPyS accessible to G proteins or somehow facilitates the exchange of guanine nucleotides remains to be elucidated.

Whatever the exact mechanism underlying the activation of G proteins by GTPyS in depolarized cells may be, the present findings provide a simple method for activating G proteins in intact cells without permeabilization. Cells remain fully viable after a transient exposure to depolarizing K⁺ concentrations (at least up to 1 h, the longest incubation tested), which allows study of long-term effects of G protein activation, such as mitogenic effects. We show in this report that a transient exposure of Go-arrested CCL39 cells to GTPyS under depolarizing conditions: 1) is not sufficient by itself to induce a significant mitogenic response, 2) does not alter the subsequent response to α-thrombin, a mitogen known to activate G protein-mediated pathways in CCL39 cells (2, 12, 24, 41), but 3) markedly potentiates the mitogenic effects of FGF, a growth factor known to activate a receptor-tyrosine kinase (10). The amplitude of this potentiation is dependent on both the concentration of GTPyS used during the pretreatment, with a maximal effect at 2 mM, and on the time of pretreatment, with a maximal effect after 60 min when tested at 1 mM GTPyS. Very similar effects can be obtained with GDPβS, except that a longer exposure is required since this analog becomes stimulatory only after a lag phase of 15–20 min.

How do these long-term effects of GTPyS and GDPβS compare with their early effects on G proteins in depolarized CCL39 cells? It is noteworthy that the same delay of 15–20 min is observed for the activation of PLC by GDPβS, whereas the activation by GTPyS is immediate. Moreover, a 60-min exposure to GTPyS or GDPβS, which results in the same mitogenic stimulation, leads to an equivalent stimulation of...
PLC and a similar dual regulation of adenylate cyclase. Comparison of the dose-response curves for the primary and long-term effects of GTPγS and GDPβS suggests, however, that the mitogenic effects of these two nucleotides are very unlikely to be accounted for by the sole G, mediated inhibition of adenylate cyclase, despite the apparent importance of G, activation in mitogenesis (6, 8). Indeed, adenylate cyclase is maximally inhibited after a 60-min exposure to 0.5 mM GTPγS or GDPβS, whereas this concentration gives only half-maximal potentiation of FGF-induced DNA synthesis. In fact, in the range of concentrations that are optimal for DNA synthesis, GTPγS and (GDPβS) exert both inhibitory (through G,) and stimulatory (through G, and protein kinase C) effects on adenylate cyclase. These opposite effects roughly compensate each other when the cyclase is activated by forskolin, whereas the G, mediated stimulation prevails in the absence of forskolin, the conditions used for measurement of DNA synthesis. It is therefore extremely unlikely that a decrease in cAMP mediates the mitogenic effects of the guanine nucleotide analogs. Other cellular effects of G, are presumably involved, possibly including its effects on PLC, since it is still unclear whether G, and Gp activities are mediated by completely distinct entities.

It is interesting to note that both the short-term effects of GTPγS and GDPβS on G, and Gp, and their long-term effects on mitogenesis are suppressed by pertussis toxin. This observation does not support the commonly accepted view that pertussis toxin-catalyzed ADP-ribosylation leads to uncoupling of the G protein from the receptor, without impairing its direct activation by stable guanine nucleotides (42), except if one assumes that in cells agonist free receptors can interact with G proteins and facilitate guanine nucleotide exchange, a possibility suggested by recent findings obtained with Gi2 and G protein. This observation is believed to occur without involving receptor-G protein interaction (44). Thus, the exact mechanisms underlying inactivation of G proteins by pertussis toxin remain to be clarified.

Our data indicate that at least one pertussis toxin-sensitive G protein is involved in the stimulation of DNA synthesis by GTPγS and GDPβS, which is very likely to be the one activated by α-thrombin, as supported by the following observations: 1) a pretreatment with GTPγS protects thrombin-induced mitogenesis against pertussis toxin, 2) the synergistic effects of GTPγS and thrombin on FGF response are similarly inhibited by pertussis toxin, and 3) GTPγS does not synergize with thrombin. These results, however, do not further our information on the identity and the function of the G protein(s) essential for mitogenesis. But whatever the nature of this pertussis toxin-sensitive signal be, it is worth noting that this signal is much more critical when thrombin is used alone than when it is used in synergy with FGF, as judged by the shift in the dose-response curve for pertussis toxin inhibition (half-maximal inhibition is obtained with ~0.1 ng/ml in the first case and >10 ng/ml in the second one). This observation supports the view that the pertussis toxin-sensitive signal is only one of the multiple signals evoked by thrombin and if this signal is crucial when thrombin is used as the sole mitogen, it can be replaced in the presence of FGF by a tyrosine kinase-induced event. Inhibition of DNA synthesis by pertussis toxin has also been reported to be reversed by tyrosine kinase-activating growth factors in other cell systems (4, 7).
progression, as discussed recently by Pandiella et al. (50). Such a subtle balance between the various signals is certainly difficult to obtain by direct and rather unspecific activation of G proteins with GTPγS, but it might be achieved when G protein activation is finely controlled by specific interactions with receptors.

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