Agonist-sensitive binding of a photoreactive GTP analog to a G-protein α-subunit in membranes of HL-60 cells*

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Myeloid-differentiated HL-60 cells were used to study the activation of G-proteins by receptor agonists. Following incubation of membranes with the photoreactive GTP analog. [α-^{32}P]GTP azidoanilide, and subsequent exposure to ultraviolet light (254 nm), photolabeling of 40 kDa proteins comigrating with the G_{i2} α-subunit was observed. Photolabeling in the absence or presence of the chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanin (FMLP), absolutely required Mg^{2+}; FMLP stimulated photolabeling at all Mg^{2+} concentrations employed (up to 30 mM). Addition of GDP (3–50 μM) reduced basal photolabeling to a greater extent than photolabeling stimulated by FMLP. FMLP did not stimulate photolabeling of proteins modified by pertussis toxin. Leukotriene B_4 and C5a also stimulated photolabeling of 40 kDa proteins. The results indicate that (i) the major G-protein in HL-60 cells, G_{i2}, requires Mg^{2+} for basal and receptor-stimulated activity, (ii) effective receptor-mediated activation of G-proteins is observed at mM concentrations of Mg^{2+}, and (iii) receptor agonists apparently reduce the affinity of G-proteins for GDP.

Chemoattractant; Guanine nucleotide-binding protein; Photoaffinity labeling; (HL-60 cell)

1. INTRODUCTION

Most extracellular signals activate receptors coupled to regulatory heterotrimeric guanine nucleotide-binding proteins (G-proteins) which are attached to the inner face of the plasma membrane [1]. In order to identify G-proteins stimulated by a given receptor agonist, we combined receptor agonist-sensitive photolabeling of membrane proteins with the hydrolysis-resistant GTP-analog, [α-^{32}P]GTP azidoanilide ([α-^{32}P]AA-GTP) [2,3], with separation of membrane proteins by high-resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of urea.

As a model system, we used membranes from myeloid-differentiated HL-60 cells. In this cell line, similar to human neutrophils, a variety of cellular functions, e.g. superoxide production and activation of a phospholipase C, are initiated by binding of chemoattractants to specific cell surface receptors which are coupled to pertussis toxin-sensitive G-proteins [4–6]. As was shown by Northern and Western blot analyses, HL-60 cells express the pertussis toxin-sensitive G-proteins, G_{i2} and G_{i3}, with α-subunits of 40 and 41 kDa, respectively, but not the pertussis toxin-sensitive G-proteins, G_{i1} and G_o [7,8]. Accordingly, two pertussis toxin substrates of 40 and 41 kDa were purified from membranes of HL-60 cells [9].

We report here on the stimulatory effects of the chemoattractants, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B_4 (LTB_4) and the N-terminal active proteolytic fragment of the complement component C5 (C5a), on photolabeling with [α-^{32}P]-AA-GTP of a membrane protein comigrating with the G_{i2} α-subunit.

2. MATERIALS AND METHODS

FMLP, LTB_4 and C5a were purchased from Sigma (Deisenhofen, FRG); nucleotides were from Boehringer Mannheim (Mannheim, FRG); urea was from Bio-Rad (München, FRG). Sources for other materials have been cited [10–12].

Culture, differentiation with dimethyl sulfoxide and fractionation of HL-60 cells were peformed as described previously [13]. Protocols for culture of rat pituitary (GH_3) cells and preparation of membranes are given in [14].

[α-^{32}P]AA-GTP was synthesized by incubation of [α-^{32}P]GTP with azidoaniline [2,15] and purified by ion-pairing chromatography on a C_{18} column, using volatile solvents [16]. Details of synthesis and purification will be published elsewhere [12]. [α-^{32}P]AA-GTP was specifically incorporated into α-subunits of G-proteins purified from porcine brain (G_i, G_o) or human erythrocytes (G_i, G_s). Photolabeling of G-protein α-subunits was dependent on irradiation with ultraviolet light and abolished by adding GTP but not by adding ATP in a 1000-fold excess over [α-^{32}P]AA-GTP to the reaction mixture.

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For photolabeling of proteins with [α-^{32}P]AA-GTP, membranes from HL-60 (or GH_3 cells) (50 μg of protein/assay tube) were incubated at 30°C in a buffer consisting of 0.1 mM EDTA, 1 (or 5) mM $MgCl_2$, 1 mM benzamidine, 10 mM NaCl, 3 μM GDP (or no GDP) and 30 mM Hepes (pH 7.4); variations from this buffer composition (concentrations of $MgCl_2$, GDP) are indicated in the figure legends (figs.2–3). After 3 min of preincubation with receptor agonists, samples were incubated for another 3 min with [α-^{32}P]AA-GTP (15 kBq/tube) at a final concentration of 5–30 nM. The final assay volume was 60 μl. The reaction was stopped by cooling the sample on ice. After centrifugation at 12 000 × g for 5 min at 4°C (for removal of unbound [α-^{32}P]AA-GTP), the obtained membrane pellets were resuspended in 60 μl of a modified GDP-free incubation buffer supplemented with 2 mM DTT. Membrane suspensions were then irradiated for 10 s at 4°C with an ultraviolet lamp (254 nm, 150 W) from a distance of 3 cm. Membranes were again centrifuged, and pellets were dissolved in sample buffer [17].

SDS PAGE [17] and autoradiography of gels were performed as described [10,11] with the following modifications. After SDS PAGE and autoradiography, the 40 kDa regions of lanes of dried gels were cut out and shaken in 1 ml of 30% (v/v) H_2O_2 for at least 1 h. Incorporated radioactivity was counted after addition of 5 ml of scintillant.

Immunoblotting of proteins was performed as described [10]. Nitrocellulose filters were incubated with antisera generated against synthetic peptides corresponding to confined regions of G-protein α-subunits. The sequences of peptides used for immunization were (one-letter code):
(C)GAGESGKSTIVKQMK (α_{common} peptide), (C)NLREDGEKAAREV ($\alpha_{i\text{-}common}$ peptide), (C)TGANKYDEAAS (α_{i2} peptide), and (C)NLKEDGISAAKDVK (α_o peptide). Each peptide contained an additional cysteine residue (in parentheses) at the amino terminus in order to facilitate cross-linking to keyhole limpet hemocyanine. The synthesis of peptides, their coupling to keyhole limpet hemocyanine, immunization of rabbits and the characterization of antisera generated againt these peptides (with G-proteins purified from various tissues) have been described elsewhere [14, 18–20]. For detection of bound antibodies, nitrocellulose filters were incubated for 1 h at room temperature with goat antirabbit IgG coupled to alkaline phosphatase. Immunoreactive bands were visualized by the color reaction of Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate catalyzed by alkaline phosphatase [21].

[α^{32}P]GTP was synthesized according to Johnson and Walseth [22]. Protein was determined according to Lowry et al. [23], using bovine serum albumin as a standard. Data reported here are representative for data obtained in two or more independent experiments.

3. RESULTS

In membranes from HL-60 cells, [α-^{32}P]AA-GTP was incorporated into proteins migrating as 40 kDa species in urea-containing SDS gels. Typically, autoradiograms showed a band with an intense lower and a faint upper portion (fig.1). In the absence of urea, the photolabeled proteins migrated slightly faster, and the autoradiogram showed a sharp, narrow band (not shown). In order to elucidate the identity of the photolabeled 40 kDa proteins, membranes from HL-60 cells and – for comparison – membranes from GH_3 cells were incubated with [α-^{32}P]AA-GTP. Following SDS-PAGE in the presence of urea, proteins were blotted onto nitrocellulose filters, which were subjected to autoradiography and subsequently incubated with different antisera; filter-bound antibodies were visualized by a color reaction (see fig.1). The α_{common} peptide antiserum which recognizes all G-protein α-subunits except that of G_z, the $\alpha_{i\text{-}common}$ peptide antiserum which recognizes the various G_i α-subunits and the α_{i2} peptide antiserum which only recognizes the G_{i2} α-subunit [14, 18–20] detected a 40 kDa protein comigrating with the main photolabeled protein. The data indicate that the photolabeled proteins represent the G_{i2} α-subunit. This assumption is supported by the finding that the α_{common}, $\alpha_{i\text{-}common}$ and α_{i2} peptide antisera reacted with a protein with an indistinguishable mobility in membranes from GH_3 cells. In membranes from HL-60 cells, the α_{common} and $\alpha_{i\text{-}common}$ peptide antisera but not the α_{i2} peptide antiserum also reacted with a 41 kDa protein probably representing the G_{i3} α-subunit (see section 1). This 41 kDa protein, which is difficult to recognize on the shown photograph but clearly detectable on the stained nitrocellulose filters, comigrated with the faint upper portion of the 40 kDa band of the autoradiogram. The α_o peptide antiserum which recognizes the various forms of G_o α-subunits but not G_i α-subunits did not react with a protein in membranes from HL-60 cells (see section 1). In membranes from GH_3 cells which have been shown to possess G_o [14], the α_o peptide antiserum reacted with a 39 and a 40 kDa protein. The mobility of the 40 kDa protein recognized by the α_o peptide antiserum was indistinguishable from that of the protein recognized by the α_{i2} peptide antiserum. In contrast to autoradiograms from experiments with HL-60 cells, autoradiograms from experiments with GH_3 cells showed two completely resolved photolabeled proteins of 39 and 40 kDa. These proteins comigrated with those detected by the α_o peptide antiserum (39 and 40 kDa) and the α_{i2} peptide antiserum (40 kDa).

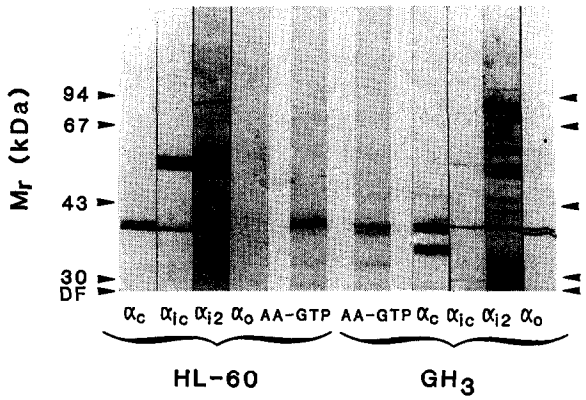

Fig.1. G-protein α-subunits in membranes from HL-60 and GH_3 cells. Membrane proteins were photolabeled with [α-^{32}P]AA-GTP and separated by SDS PAGE in the presence of urea as described in section 2. Separated proteins were transferred to nitrocellulose filters, and the filters were incubated with different peptide antisera. Filter-bound antibodies were visualized by a color reaction. Shown are autoradiograms of the filters (AA-GTP) and the stained filters (α_c, α_{common} peptide antiserum, α_{ic}, $\alpha_{i\text{-}common}$ peptide antiserum, α_{i2}, α_{i2} peptide antiserum, α_o, α_o peptide antiserum). DF, dye front.

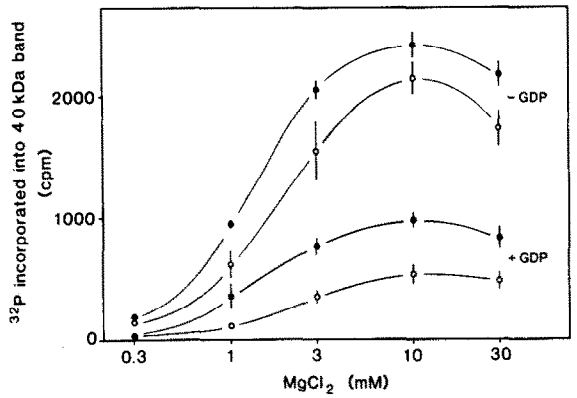

Fig.2. Influence of $MgCl_2$ on the incorporation of [α-^{32}P]AA-GTP into 40 kDa proteins of membranes from HL-60 cells. Membranes from HL-60 cells were incubated with [α-^{32}P]AA-GTP in the absence (open circles) and presence (closed circles) of 1 μM FMLP, without or with 3 μM GDP as indicated in the figure and with increasing concentrations of $MgCl_2$ (abscissa). Photolabeling and SDS-PAGE (in the presence of urea) was performed as described in section 2. The 40 kDa region of lanes of dried gels was counted for radioactivity (ordinate). Values are mean values of triplicates ± SD.

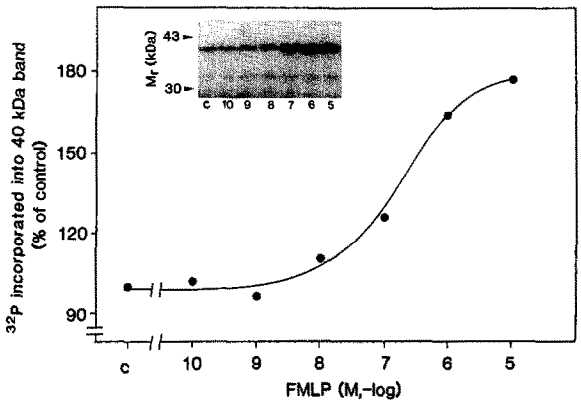

Fig.4. Concentration-response curve for FMLP with respect to [α-^{32}P]AA-GTP incorporation into 40 kDa proteins of membranes from HL-60 cells. Experimental conditions were as described in section 2. Concentrations of FMLP are indicated on the abscissa, the radioactivity incorporated into 40 kDa proteins (in percent of control values obtained in the absence of FMLP) on the ordinate. Values are mean values of triplicates. The inset shows an autoradiogram of a representative gel; c, control.

The amount of [α-^{32}P]AA-GTP incorporated into the 40 kDa proteins in membranes from HL-60 cells increased constantly over a period of 10 min of incubation (data not shown) and was stimulated by the chemoattractant, FMLP. In order to characterize the stimulatory effect of the receptor agonist and to establish experimental conditions favorable for agonist-sensitive photolabeling, the effects of Mg^{2+} and GDP were studied (fig.2). Free Mg^{2+} was indispensable to obtain detectable photolabeling with [α-^{32}P]AA-GTP. Mg^{2+}, employed up to 10 mM, concentration-dependently stimulated photolabeling; a higher Mg^{2+} concentration (30 mM) was slightly inhibitory. The stimulatory effect of FMLP was observed at all tested Mg^{2+} concentrations. The effect of Mg^{2+} was qualitatively not altered by the addition of GDP (3 μM), which reduced photolabeling in the absence of FMLP more (75–80%) than FMLP-stimulated photolabeling (60–65%). Thus, addition of GDP resulted in an increased ratio of stimulated over unstimulated photolabeling. This is also evident from the experiment shown in fig.3. In the presence of 1 mM $MgCl_2$, GDP concentration-dependently inhibited to a different extent basal photolabeling and photolabeling stimulated by FMLP. Stimulation of photolabeling by FMLP in the absence of GDP and at GDP concentrations of 3 μM and above, amounted to 120% and

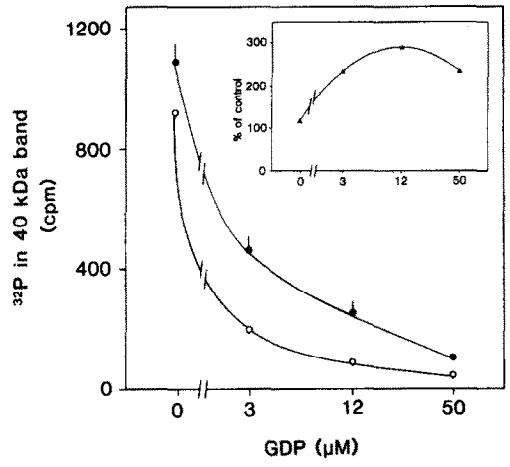

Fig.3. Influence of GDP on incorporation of [α-^{32}P]AA-GTP into 40 kDa proteins of membranes from HL-60 cells. Membranes were incubated with [α-^{32}P]AA-GTP in the absence (open circles) and presence (closed circles) of FMLP (1 μM), with 1 mM $MgCl_2$, and increasing concentrations of GDP (abscissa). Photolabeling and SDS PAGE (in the presence of urea) was performed as described in section 2. The 40 kDa region of lanes of dried gels was counted for radioactivity (ordinate). Values are mean values of triplicates ± SD. The inset shows the stimulatory effect of FMLP in percent of control values obtained in the absence of FMLP.

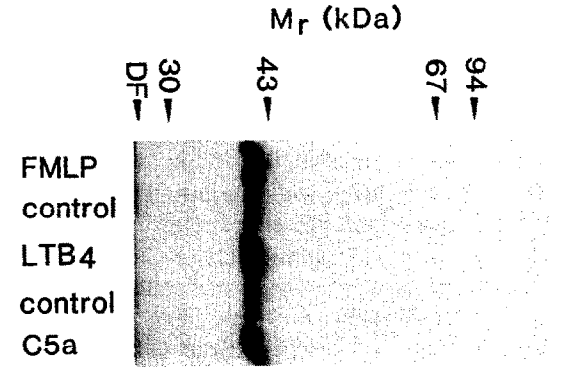

Fig.5. Effects of FMLP, C5a and LTB_4 on [α-^{32}P]AA-GTP incorporation into membrane proteins from HL-60 cells. Experimental conditions were as described in section 2. Chemoattractants were employed at a concentration of 1 μM. Shown is the autoradiogram of an SDS gel. DF, dye front.

200–300% of the appropriate controls, respectively (see inset to fig.3).

A concentration–response curve for FMLP was elaborated in the presence of 1 mM $MgCl_2$ and 3 μM GDP (fig.4). For maximal stimulation, relatively high concentrations of FMLP were necessary, similar to those required for maximal stimulation of cholera toxin-catalyzed ADP-ribosylation in membranes of HL-60 cells [24] and for maximal stimulation of GTPase activity in neutrophil membranes [25]. FMLP did not stimulate photolabeling of proteins ADP-ribosylated by pertussis toxin (data not shown). Platelet-activating factor and adrenaline did not influence photolabeling of membrane proteins.

Fig.5 shows the effects of FMLP, LTB_4 and C5a on photolabeling of membrane proteins. These chemoattractants, each employed at a concentration of 1 μM, stimulated incorporation of [α-^{32}P]AA-GTP into the 40 kDa proteins to a similar extent (about 2- to 3-fold).

4. DISCUSSION

In this report, we describe an approach to study the interaction of activated receptors with G-proteins. The application of this method does not depend on solubilisation of membrane proteins or on reconstituted systems, e.g. purified proteins incorporated into phospholipid vesicles. Instead, it allows to study the interaction of signal transduction components 'in situ', i.e., within the native plasma membrane.

Applying this method to membranes from HL-60 cells, we found that various chemoattractants (FMLP, LTB_4 and C5a) stimulate incorporation of [α-^{32}P]AA-GTP into 40 kDa proteins. Following incubation of membranes with [α-^{32}P]AA-GTP and irradiation with ultraviolet light, we were able to detect two resolved G_i α-subunits (presumably the α-subunits of G_{i2} and G_{i3}) on nitrocellulose filters by immunological means, whereas a resolution of the comigrating photolabeled proteins was not observed on autoradiograms obtained from the same filters. The mobility of the major portion of photolabeled proteins correspond to that of the G_{i2} α-subunit. The present data, therefore, indicate that receptors for chemoattractants are functionally coupled to G_{i2}. They do, however, not exclude the possibility that chemoattractants exert their effects also via G_{i3}. Failure to demonstrate a stimulatory effect of chemoattractants on photolabeling of the G_{i3} α-subunit may be due to the apparently low levels of G_{i3} compared to that of G_{i2} and the incomplete resolution of photolabeled G_i α-subunits. Evidence for the assumption that chemoattractants act via G_{i2} *and* G_{i3} has been provided in a preliminary report by Sidiropoulos et al. [26]. This group observed that in membranes from HL-60 cells, FMLP stimulated cholera toxin-catalyzed ADP-ribosylation of two proteins of about 40 kDa provided guanine nucleotides were absent from the reaction mixture.

Other methods suitable for direct or indirect demonstration of G-protein activation in membrane preparations, e.g. measuring the activity of high-affinity GTPase or adenylyl cyclase, are routinely performed with a nucleoside triphosphate-regenerating system to avoid formation of nucleoside diphosphates. These experimental conditions have hampered studies designed to examine the role of GDP in G-protein activation. Our data suggest that GDP which is a constituent of the cytosol may play an important role in the modulation of G-protein activity. We provide evidence that – at least in the presence of [α-^{32}P]AA-GTP at nanomolar concentrations – GDP at μM concentrations promotes the stimulation of G-proteins by receptor agonists by inhibiting binding of GTP or its analogs to α-subunits more efficiently in the absence of receptor agonists than in their presence. The relative stimulation by chemoattractants in HL-60 cells as well as by hormones, neurotransmitters and drugs in other cell systems, e.g. neuroblastoma × glioma hybrid cells (108CC15) [27], pituitary GH_3 cells and rat insulinoma (RINm5F) cells (unpublished), was greatest at GDP concentrations ranging from 3 to 50 μM. This promotion of agonist effects by GDP is consistent with the hypothesis that the relative affinities of G-proteins for guanine nucleotides (GDP vs GTP) change during activation [28]. The unstimulated G-protein may possess a relatively high affinity for GDP, whereas the activated G-protein preferably binds GTP. It is tempting to speculate that in the intact cell GDP is responsible for a low basal activity of G-proteins, thus allowing an effective activation of G-proteins via receptors. To test this hypothesis more thoroughly, experiments have to be performed with physiologically relevant concentrations of GDP and GTP or its analogs.

Incorporation of [α-^{32}P]AA-GTP into 40 kDa proteins, as determined in the absence or presence of GDP, increased over a wide range of Mg^{2+} concentrations (up to 10 mM); similar observations have been made with respect to binding of a GTP analog to the purified cholera toxin-sensitive G-protein, G_s, which confers hormonal stimulation to adenylyl cyclase [29]. Higashijima et al. [30] working with the purified pertussis toxin-sensitive G-protein, G_o, suggested that activation of G-proteins by Mg^{2+} applied at mM concentrations is due to an increased dissociation of (endogenous or exogenous) GDP bound to the α-subunit, thus allowing fast binding of GTP and its analogs to the 'empty' α-subunit. Our findings are consistent with this hypothesis.

An effective stimulation of photolabeling by FMLP was observed at all Mg^{2+} concentrations tested. The present data differ from those obtained for the hormonal stimulation of adenylyl cyclase in liver membranes [31]. In this system, receptors apparently act as

'Mg^{2+} switches', since glucagon dramatically lowers the Mg^{2+} concentration required for full activation of the enzyme. Whereas this finding refers to the stimulatory G-protein, G_s, it may not be applicable to G_i-type G proteins.

Only after prolonged exposure time of films, we observed photolabeling of proteins with the mobility of G_s α-subunits (42–45 kDa). This may be due to the fact that the level of G_s in membranes from most tissues, particularly from brain and leucocytes, appears to be low in comparison to those of pertussis toxin-sensitive G-proteins. Nevertheless, photolabeling of G-protein α-subunits appears to be a valuable tool to investigate, within the native plasma membrane, regulation of G-proteins by various ligands and their interaction with activated receptors. Further studies are directed towards the identification of G-proteins or G-protein subtypes activated by a given receptor.

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