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Desensitization Uncouples the Formyl Peptide Receptor-Guanine Nucleotide-Binding Protein Interaction in HL60 Cells

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SUMMARY

Formyl peptide receptors on differentiated HL-60 cells were desensitized to formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated superoxide production in a concentration-dependent manner, similar to that previously described for neutrophils. Membranes isolated from desensitized (DM) and normal (NM) HL-60 cells were used to compare receptor numbers and affinities between NM and DM and compare the ability of receptors on DM and NM to interact normally with their guanine nucleotide regulatory proteins (G proteins). Exposure of differentiated HL-60 cells to 10^{-7} M FMLP for 10 min before membranes were isolated resulted in a 75% reduction in receptor number, without alteration of dissociation constants. The remaining receptors on DM did not interact normally with their G proteins, as demonstrated by 1) the failure of guanine nucleotides to alter FMLP binding, 2) the inability of FMLP to stimulate guanosine-5′-*O*-(3-thiotriphosphate) binding, and 3) the attenuation of FMLP stimulation of GTPase activity. These results were not due to a reduction in G protein content of DM, as determined by Western blot analysis with an antibody that recognized α_{40} and by pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of membrane G proteins in NM and DM. The failure of FMLP receptors on DM to interact with their G proteins was not due to differences in receptor number between NM and DM. Increasing the Mg^{2+} concentration partially restored the FMLP receptor-G protein interaction in DM. We conclude that desensitization of the formyl peptide receptor is associated with both loss of membrane receptors and a functional alteration in the receptor-G protein interaction, which can be partially reversed by increased concentrations of Mg^{2+}.

One mechanism by which cells are able to regulate their responsiveness to membrane receptor stimulation is desensitization, that is, a loss of responsiveness despite continued presence of the agonist and attenuation of the response to subsequent agonist exposure. Desensitization has been extensively studied in two receptors, the photoreceptor rhodopsin and the βAR, which are coupled to their effector enzymes by G proteins. Desensitization of both receptors is a multistep process that results in uncoupling of receptors from effector enzymes.

Binding of an agonist to the βAR uncouples the receptor from adenylate cyclase (reviewed in Refs. 1–3) by a process dependent on receptor-specific kinase-induced phosphorylation of the βAR (4–6). Uncoupling of the βAR may result from physical sequestration of the receptor away from G proteins and adenylate cyclase or from a functional alteration in the receptor, rendering it incapable of interacting with G proteins. βAR desensitization is accompanied by internalization of 60–95% of surface receptors (7–10), unaccompanied by internalization of G proteins or adenylate cyclase (8). Both internalized and surface βAR from desensitized cells have been reported to interact normally with their G proteins. Reconstitution of internalized βAR from desensitized cells with normal G proteins and adenylate cyclase restores hormone-stimulated cyclase activity (11). Strasser and Lefkowitz (12) have reported that treatment of desensitized S49 cells with the fusogen polyethylene glycol restores hormone-stimulated enzyme activity. These studies suggest that sequestration is the primary mechanism of desensitization. On the other hand, desensitization can be detected before internalization of βAR (7, 9, 13), and βAR on desensitized cells have been shown not to interact normally with their G proteins (13–15). Prevention of receptor internalization by pretreatment with concanavalin A did not block desensitization of βAR in astrocytoma cells (16). Bouvier *et al.* (17) have recently shown that removal of βAR phosphorylation sites delays desensitization without altering internalization. Therefore, conflicting data exist regarding the mechanism of desensitization of βAR.

The mechanism of desensitization of rhodopsin is clearly the result of a functional impairment of the receptor-G protein

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ABBREVIATIONS: βAR, β-adrenergic receptor; FMLP, formyl-methionyl-leucyl-phenylalanine; G protein, guanine nucleotide regulatory protein; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); DM, membranes from desensitized cells; NM, membranes from normal cells.

interaction, because internalization does not occur. Desensitization of rhodopsin results from a light-activated alteration in the receptor, leading to phosphorylation by a receptor-specific kinase (18, 19). The phosphorylated receptor binds a 48-kDa protein, arrestin, which interferes with the interaction between rhodopsin and its G protein, transducin. These studies suggest that a functional alteration of the receptor-G protein interaction may be a mechanism of desensitization common to G protein-regulated receptors.

The formyl peptide receptor expressed by neutrophils and other phagocytic cells belongs to the family of receptors coupled to effector enzymes by G proteins (20, 21). These receptors undergo desensitization in the presence of an agonist such as FMLP, as measured by the loss of stimulated superoxide production (22). Similar to βAR desensitization, formyl peptide receptors are internalized during desensitization (23–25). Although the loss of cellular response to formyl peptides is greater than the loss of receptors would predict (26), inhibition of internalization by dihydrocytochalasin B prevents desensitization (27). Therefore, it remains to be established whether desensitization of the formyl peptide receptor functionally alters the receptor-G protein interaction.

In the present study, the formyl peptide receptor on the human promyelocytic cell line HL60 was used to determine whether desensitization of the formyl peptide receptor is accompanied by uncoupling of the receptor-G protein interaction. Our results indicate that formyl peptide receptors are lost from the cell surface and the remaining surface receptors are functionally uncoupled from their G proteins. High concentrations of Mg^{2+} can partially reverse this uncoupling. These results support the hypothesis that inhibition of the receptor-G protein interaction is a mechanism of desensitization common to many diverse receptors.

Materials and Methods

Reagents. FMLP was obtained from Sigma (Deisenhofen, FRG). GDP, GTP, and GTPγS were obtained from Boehringer (Mannheim, FRG). [^{3}H]FMLP (55–60 Ci/mol) and [^{35}S]GTPγS (1200–1300 Ci/mol) were from DuPont-New England Nuclear (Dreieich, FRG). [γ-^{32}P]GTP and [^{32}P]NAD were prepared as previously described (28, 29). Pertussis toxin were prepared from supernatents of *Bordetella pertussis* suspensions that were kindly provided by Dr. F. Blackkolb (Behringwerke, Marburg), as previously described (30).

Cell culture and membrane preparation. HL60 cells were grown in suspension culture in RPMI 1640 that was supplemented with 10% horse serum, 1% nonessential amino acids, 2 mM *l*-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere at 37° in 8% CO_2. To induce myeloid differentiation, cells were seeded at a density of 10^6 cells/ml and were cultured for 5 days in medium containing 1.25% dimethyl sulfoxide (31). HL60 cell membranes were prepared as previously described, except that centrifugation through Percoll gradients was omitted (32).

FMLP binding. FMLP binding was determined in a reaction mixture (100 μl) containing 20 mM Tris, 1 mM EDTA, $MgCl_2$ at the indicated concentration, and [^{3}H]FMLP (0.02 to 0.04 μCi/tube unless otherwise indicated). The reaction was initiated by addition of 15 to 25 μg of membrane protein to each tube. The reaction was terminated after 30 min at 25° by rapid filtration through GF/C Whatman filters, followed by four washes with 2.5 ml of ice-cold buffer containing 50 mM triethanolamine/HCl, 5 mM $MgCl_2$, and 1 mM EDTA. Filters were dried and counted in a liquid scintillation counter. Membrane protein was determined according to the method of Bradford (33), using bovine IgG as the standard. Specific binding was calculated as pmol of FMLP/mg of membrane protein by subtracting the amount of [^{3}H]FMLP bound in the presence of 10 μM FMLP from the total [^{3}H]FMLP bound.

Binding parameters were estimated using a nonlinear least squares curve-fitting procedure (SCTFIT). The varience function used by SCTFIT to assign weights to data points is $\text{var}(y) = a_0 + a_1 \times y^{a_2}$, where y is the amount of radioligand bound to the membrane. The values used for a_0, a_1, and a_2 (2.09×10^{-6}, 1.58×10^{-3}, and 1.53, respectively) were obtained from analyzing 130 triplicate determinations of [^{3}H]FMLP binding to HL-60 cell membranes (34). Testing for statistically significant resolution of two receptor subtypes was performed by comparing the residual variance of the fit obtained when the data were analyzed according to a model for a single class or two classes of binding sites, using a partial F test (35).

GTPγS binding. GTPγS binding was determined in a reaction mixture (100 μl) containing 50 mM triethanolamine/HCl, pH 7.4, 1 mM dithiotreitol, 1 mM EDTA, $MgCl_2$ at the indicated concentration, 150 mM NaCl, 0.5 μM GDP, and [^{35}S]GTPγS (0.02 to 0.04 μCi/tube). The reaction was initiated by addition of 2 to 10 μg of membrane protein to each tube. The reaction was terminated, after 20 min at 30°, by rapid filtration through GF/C Whatman filters, followed by four washes with 2.5 ml of ice-cold buffer that contained 50 mM triethanolamine/HCl and 5 mM $MgCl_2$. Filters were dried and counted in a liquid scintillation counter. Specific binding was calculated as pmol of GTPγS bound/mg of membrane protein by subtracting [^{35}S]GTPγS bound in the presence of 10 μM GTPγS from the total [^{35}S]GTPγS bound.

GTPase assay. Hydrolysis of [γ-^{32}P]GTP (0.02 to 0.05 μCi/tube) was determined in a reaction mixture (100 μl) that contained 2 to 10 μg of membrane protein, 50 mM triethanolamine/HCl, pH 7.4, 1 mM dithiotreitol, 0.1 mM ATP, $MgCl_2$ at the indicated concentration, 1 mM EDTA, 100 mM NaCl, 0.1 μM GTP, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, and 0.2% bovine serum albumin. Reactions were performed for 10 min at 30° in the absence or presence of FMLP. Reactions were terminated and high affinity GTPase activity was determined as previously described (36). Results were expressed as pmol of free P_i generated/min/mg of protein.

O_2^- assay. O_2^- generation was measured by cytochrome *c* (type VI; Sigma) reduction. All experiments were performed in duplicate, in a volume of 1 ml, in microcentrifuge tubes that contained 4×10^6 cells, 80 mM cytochrome *c*, and the indicated concentration of FMLP or NaF. To produce desensitization, cells were exposed to various concentrations of FMLP in the absence of cytochrome *c*. After 10 min, cytochrome *c* was added to each tube, followed by the addition of FMLP or NaF. Ten minutes after addition of the second stimulus, tubes were centrifuged at 10,000 rpm for 30 sec. The amount of reduced cytochrome *c* was calculated from the absorbance at 550 nm of the supernatant, using the extinction coefficient of 2.1 $M^{-1}cm^{-1}$. O_2^- generation was expressed as nmol of reduced cytochrome $c/4 \times 10^6$ cells.

Pertussis toxin-stimulated ADP ribosylation. Pertussis toxin was activated by dilution with an equal volume of 40 mM dithiotreitol and was incubated for 10 min at 30°. Membranes (40–100 μg of protein) were incubated for 60 min at 37° in a volume of 50 μl that contained 10 mM potassium phosphate buffer, pH 7.5, 2.5 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 10 mM arginine, 100 μg/ml activated toxin, and 1 μM [^{32}P]NAD (20 μCi/nmol). After this incubation, 50 μl of detergent buffer was added, and the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiography of the dried gels was performed. In some experiments, the labeled G protein was cut from the gels and solubilized overnight in 1 ml of 30% H_2O_2 at 55°, and 1 ml of 30% H_2O_2 and 18 ml scintillation fluid were added before liquid scintillation counting.

Immunoblots. Immunoblotting was performed as previously described (37), using the polyclonal antibody AS6, which was kindly provided by Dr. Allen Spiegel, National Institutes of Health (Bethesda, MD).

Results

The first goal of these studies was to determine whether HL60 cells undergo desensitization to FMLP, similar to that seen with neutrophils, and to establish the concentration of FMLP at which optimal desensitization occurred. Intact HL60 cells were preincubated with various concentrations of FMLP for 10 min, at 37°, before a second 10-min period in which O_2^- production was measured in the presence of 10^{-7} M FMLP. In preliminary studies, this concentration of FMLP was found to produce maximal stimulation of O_2^- release. O_2^- production following preincubation with various concentrations of FMLP is shown in Fig. 1. The concentrations of FMLP that produced maximal and half-maximal desensitization were 10^{-7} M and 3×10^{-9} M, respectively. Desensitization with 10^{-7} M FMLP did not alter the ability of G protein activation by NaF to simulate O_2^- production (Fig. 1). In subsequent experiments, DM were prepared from cells that were incubated with 10^{-7} M FMLP for 10 min at 37° just before membrane preparation. NM were prepared from the same cell cultures, which were treated identically except for the omission of FMLP.

Because receptor internalization has been described during formyl peptide receptor desensitization (23–25), we examined receptor number and affinity in DM and NM. Binding of [^{3}H] FMLP was determined in the absence or presence of 5 mM $MgCl_2$ in addition to 1 mM EDTA. Transformation of the binding data according to the method of Scatchard (Fig. 2) was subjected to computerized nonlinear least squares curve-fitting analysis. In the absence of $MgCl_2$ ($[Mg^{2+}] = 0$ mM) a two-site model produced a significantly better fit than a one-site model. The dissociation constants of the high affinity sites (0.072 nM) and of the low affinity sites (5.34 nM) were not significantly different between DM and NM. The number of high affinity binding sites was similar in DM and NM (0.071 pmol/mg versus 0.092 pmol/mg, respectively). However, there was a marked reduction in the number of low affinity binding sites in DM (0.311 pmol/mg) compared with NM (1.464 pmol/mg). Under the conditions of the experiments performed at 5 mM $MgCl_2$ ($[Mg^{2+}] = 4$ mM), computer analysis indicated that a one-site model provided a better fit. The dissociation constants were not significantly different between the two membrane preparations (0.436 nM). Again, the number of binding sites remained significantly lower in DM (0.360 pmol/mg versus 1.988 pmol/mg, DM versus NM). Thus, the desensitization protocol resulted in about a 75% reduction in receptor sites detected on isolated membranes, without significantly altering receptor affinities.

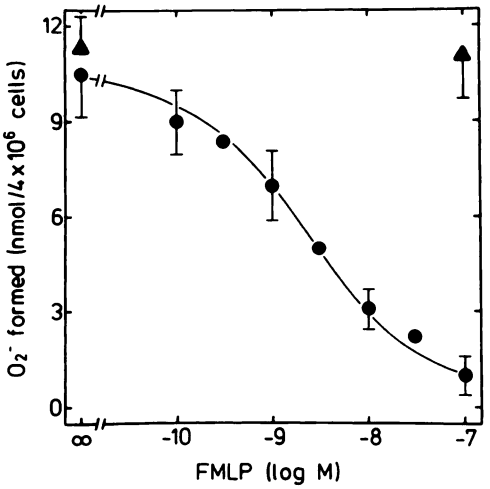

Fig. 1. Dose response of desensitization of differentiated HL60 cells to FMLP. Cells (4×10^6) were incubated with the concentration of FMLP indicated on the *abscissa*, for 10 min at 37°. Thereafter, 80 μM cytochrome *c* and 10^{-7} M FMLP (●) or 35 mM NaF (▲) were added. Following another 10-min incubation at 37°, reduced cytochrome *c* was determined spectrophotometrically. Results are expressed as the mean nmol of reduced cytochrome *c*/4 × 10^6 cells, from one to five separate experiments each performed in duplicate.

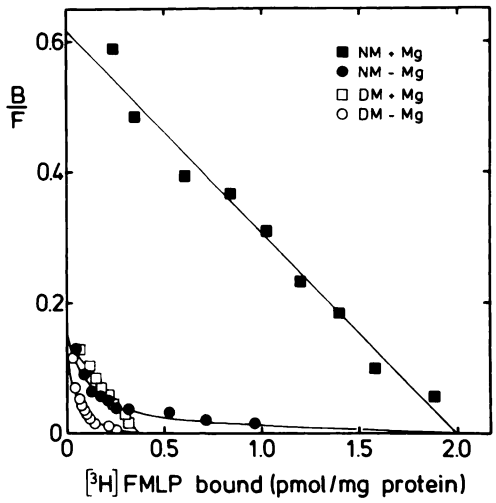

Fig. 2. Scatchard transformation of FMLP binding to NM (*solid symbols*) and DM (*open symbols*) in the presence of 1 mM EDTA and 0 mM $MgCl_2$ ($[Mg^{2+}] = 0$ mM) (*circles*) or 5 mM $MgCl_2$ ($[Mg^{2+}] = 4$ mM) (*squares*). At 0 mM Mg^{2+}, a two-site receptor model provides a better fit than a one-site model. Dissociation constants for high affinity binding sites (0.072 nM) and low affinity binding sites (5.34 nM) did not differ between NM and DM. The number of binding sites for DM versus NM was 0.071 versus 0.092 pmol/mg for high affinity sites and 0.311 versus 1.464 pmol/mg for low affinity sites. At 4 mM Mg^{2+}, a one-site model provided a better fit. Dissociation constants were not different between NM and DM (0.436 nM). The number of binding sites was 0.360 pmol/mg for DM and 1.988 pmol/mg for NM. *B/F*, bound/free.

The ability of the formyl peptide receptors remaining on the membranes of desensitized cells to interact with G proteins was examined by several methods. Guanine nucleotides have been shown to reduce the affinity of the formyl peptide receptor for its ligands (38). Therefore, the ability of various concentrations of GTPγS and GDP to inhibit FMLP binding to DM and NM was examined. Fig. 3 shows the effect of GTPγS on FMLP binding to DM and NM in the absence of Mg^{2+}. Increasing concentrations of GTPγS inhibited FMLP binding to NM, while having little or no effect in DM. Similar results were seen when GDP was used in place of GTPγS (data not shown). The functional interaction between the formyl peptide receptor and its G protein was also assessed by examining FMLP-stimulated GTPγS binding and GTPase activity in DM and NM. As shown in Fig. 4, increasing concentrations of FMLP stimulated an increased GTP-GDP exchange, as measured by GTPγS binding, in NM. On the other hand, FMLP did not enhance GTPγS binding in DM. Similarly, FMLP stimulated a dose-dependent increase in GTPase activity in NM, whereas the response in DM was markedly attenuated (Fig. 5).

One possible explanation for the failure of FMLP receptors to functionally couple with G proteins is a reduction in membrane G protein content during desensitization. This possibility

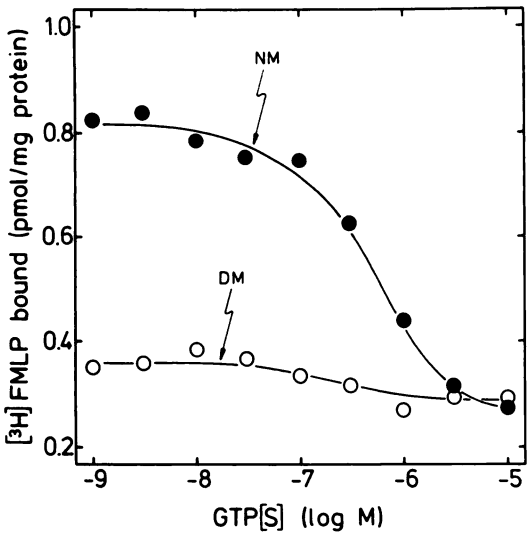

Fig. 3. Dose response of FMLP binding to increasing concentrations of GTPγS in NM (●) and DM (○). Results represent the mean of triplicate samples from one experiment that is representative of four experiments using three different membrane preparations. Experiments were performed in the presence of 1 mM EDTA and 0 mM $MgCl_2$ ($[Mg^{2+}] = 0$ mM). Results are expressed as pmol of [^{3}H]FMLP bound/mg of membrane protein.

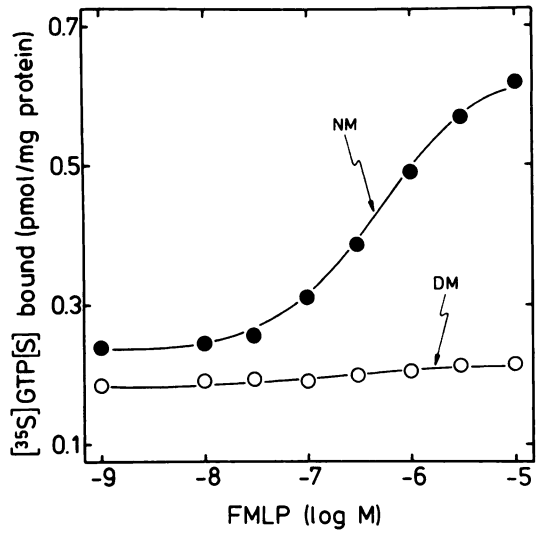

Fig. 4. Ability of FMLP to stimulate GTPγS binding in NM (●) and DM (○). Results are the mean of triplicate samples of one experiment that is representative of five experiments using three different membrane preparations. The experiments were performed in the presence of 1 mM EDTA and 1 mM $MgCl_2$ ($[Mg^{2+}] = 38.4$ μM). Results are expressed as pmol of [^{35}S]GTPγS/mg of membrane protein.

was evaluated by two methods. First, a polyclonal antibody against the C terminal decapeptide of the α subunit of transducin was used to compare quantities of the α_{40} subunit in NM and DM, by immunoblotting. As shown in Fig. 6, five membrane pairs from different cell cultures were analyzed. In two of the five some reduction in staining in DM, compared with NM, was observed, whereas staining appeared equal in the other three. Second, the ability of pertussis toxin to catalyze [^{32}P] ADP ribosylation of α_{40} was analyzed by autoradiography. As shown in Fig. 6, a small difference was seen in two of three membrane pairs. However, when gels were cut and the amount

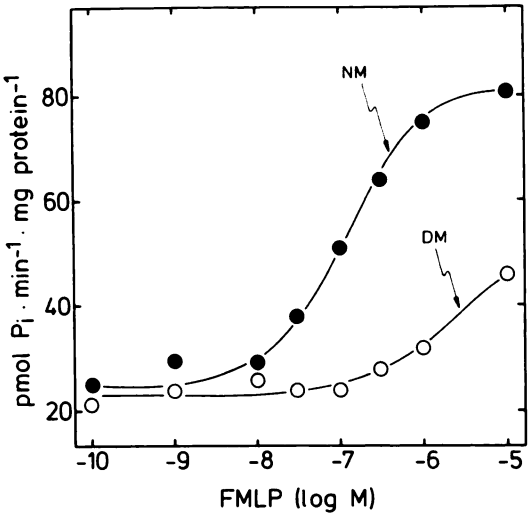

Fig. 5. Ability of FMLP to stimulate GTPase activity in NM (●) and DM (○). Results are the mean of triplicate samples of a single experiment that is representative of six experiments using four different membrane preparations. Experiments were performed in the presence of 1 mM EDTA and 1 mM $MgCl_2$ ($[Mg^{2+}] = 38.4$ μM). Results are expressed as pmol of P_i generated/min/mg of membrane protein.

of radioactivity in these bands was counted, no differences in counts were detected.

Another possible explanation for the failure to detect receptor-G protein coupling in DM is the reduction in receptor number. To evaluate this possibility, the ability of FMLP to stimulate GTPγS binding was examined in DM and NM in which the amount of membrane protein in the assay was altered so that receptor numbers would approximate each other. Because receptors were reduced by 75% in DM, DM were added to the assay at a concentration 4 times that of NM. As shown in Fig. 7, a concentration-dependent increase in GTPγS binding was stimulated by FMLP in NM, whereas binding was unchanged in DM, despite a 4-fold increase in membrane protein in the DM assays (1.2 μg/assay versus 4.8 μg/assay, NM versus DM).

Because millimolar concentrations of Mg^{2+} altered the affinity of receptors without changing receptor number, the ability of $MgCl_2$ to affect receptor-G protein interaction was examined in DM. Fig. 8 shows the effect of addition of 5 mM $MgCl_2$ ($[Mg^{2+}] = 4$ mM) on GTPγS inhibition of FMLP binding. As expected from the data shown in Fig. 2, Mg^{2+} increased FMLP binding to DM in the absence of GTPγS. The addition of millimolar concentrations of $MgCl_2$ also permitted regulation of FMLP binding by GTPγS. Addition of $MgCl_2$ also increased the potency of GTPγS in inhibiting FMLP binding, a finding also observed in NM (data not shown). The ability of FMLP to stimulate GTPγS binding and GTPase activity in DM at micromolar or millimolar concentrations of Mg^{2+} is shown in Table 1. FMLP was able to stimulate a 2-fold increase in GTPγS binding in the presence of 4 mM Mg^{2+}, whereas no increase in binding was stimulated at 38.4 μM Mg^{2+}. Similarly, both basal and FMLP-stimulated GTPase activity were enhanced in the presence of 4 mM concentrations of Mg^{2+}.

Discussion

The present study demonstrates that HL60 cells undergo a concentration-dependent desensitization to FMLP, similar to

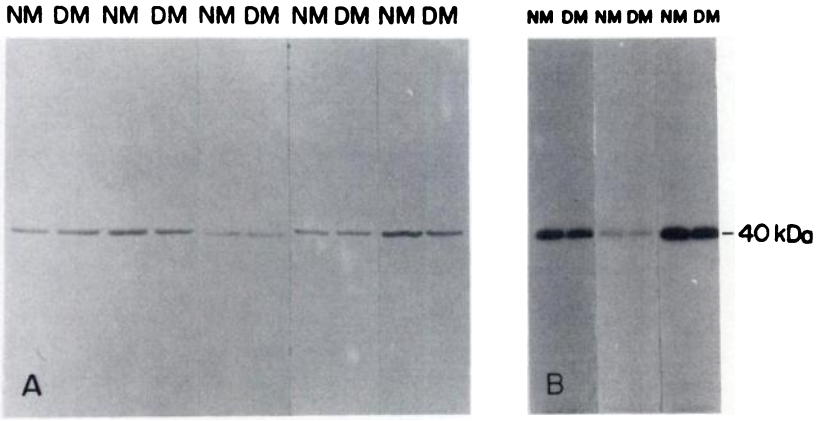

Fig. 6. A, Western blot analysis. For each pair of lanes, plasma membrane proteins (100 μg/lane) from NM and DM from HL60 cells were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and incubated with rabbit antiserum AS6 followed by goat anti-rabbit IgG-peroxidase. B, Autoradiograph of pertussis toxin-mediated ADP-ribosylation of three sets of HL60 cell DM and NM pairs.

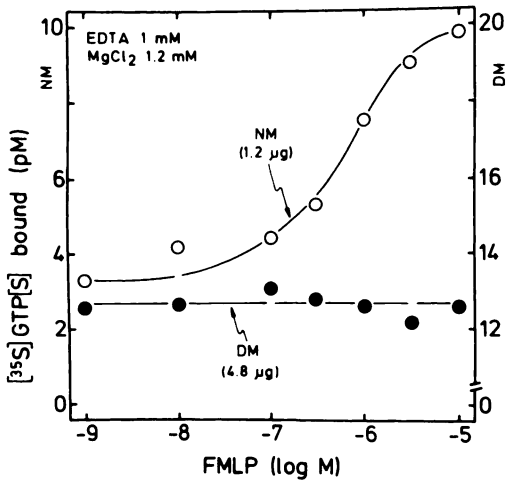

Fig. 7. Ability of FMLP to stimulate GTPγS binding in DM and NM when the receptor number in each assay was equal. NM (1.2 μg) (○) or DM (4.8 μg) (●) was added to each assay in the presence of 1 mM EDTA and 1.2 mM $MgCl_2$ ($[Mg^{2+}]$ = 207 μM). Because desensitization reduced receptor number by 75%, receptor number can be expected to be nearly equal in each of the assays. Results are expressed as pmol of [^{35}S] GTPγS bound/assay.

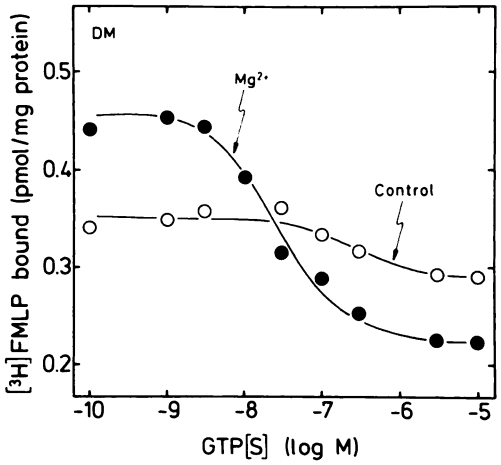

Fig. 8. Effect of Mg^{2+} on the inhibition of FMLP binding by GTPγS. Results are the mean of triplicate samples of a single experiment that is representative of three experiments with two different membrane preparations. Experiments were performed in the presence of 1 mM EDTA and 0 mM $MgCl_2$ ($[Mg^{2+}]$ = 0 mM) (○) or 5 mM $MgCl_2$ ($[Mg^{2+}]$ = 4 mM) (●). Results are expressed as pmol of [^{3}H]FMLP bound/mg of membrane protein.

that seen in neutrophils. Half-maximal desensitization of O_2^- production occurred at about 3×10^{-9} M FMLP, and maximal desensitization was produced by 10^{-7} M FMLP. Membranes from FMLP-desensitized HL60 cells were used to examine the hypothesis that desensitization of the formyl peptide receptor results in an inability of receptors to interact normally with G proteins, independent of sequestration of receptors away from G proteins. This mechanism has been clearly established as the process by which desensitization of rhodopsin occurs. Light activation of rhodopsin results in phosphorylation of the receptor, followed by binding of the protein arrestin (18, 19). These two events prevent the receptor-G protein interaction, despite the absence of physical sequestration of rhodopsin from transducin. The events that lead to desensitization of the βAR are less clear. Although the data suggest that the alteration in desensitization of βAR is localized to the receptor, the importance of physical sequestration of the receptor from G proteins and cyclase, as opposed to functional changes in the receptor that inhibit its interaction with G proteins, remains to be determined (reviewed in Ref. 1). Alteration of the phosphorylation sites on the βAR indicates that desensitization can be separated from sequestration (17). Therefore, functional changes in the receptor-G protein interaction may be common to desensitization of both βAR and rhodopsin.

TABLE 1

Effect of Mg^{2+} on FMLP-stimulated GTPγS binding and GTPase in NM and DM

Experimental conditions		GTPγS binding[a]		GTPase activity[b]	
$[Mg^{2+}]$	[FMLP]	DM	NM	DM	NM
	μM	fmol/mg		pmol/min/mg	
38.4 μM	0	400 ± 89	599 ± 59	17.6 ± 0.3	20.6 ± 1.7
	10	391 ± 51	1041 ± 51	24.1 ± 2.1	52.3 ± 5.4
4 mM	0	692 ± 55	492 ± 50	30.3 ± 0.7	47.7 ± 4.3
	10	1751 ± 147	4286 ± 145	47.2 ± 3.2	100.7 ± 4.2

[a] GTPγS binding in the presence or absence of 10 μM FMLP and 38.4 μM or 4 mM Mg^{2+}. Results are expressed as the mean ± standard deviation of GTPγS binding in three separate experiments, each performed in triplicate, in fmol/mg of membrane protein.

[b] GTPase activity in the presence or absence of 10 μM FMLP and 38.4 μM or 4 mM Mg^{2+}. Results are expressed as the mean ± standard deviation of GTPase activity in three separate experiments, each performed in triplicate, in pmol of P_i/min/mg of membrane protein.

Our study demonstrates that two processes accompany formyl peptide receptor desensitization in HL60 cells. First, formyl peptide receptors are lost from the plasma membrane. Membranes obtained from desensitized HL60 cells contained about

one fourth the formyl peptide receptors/mg of membrane protein that were found on normal membranes, whereas receptor affinities were unchanged. Although the mechanism for this loss of receptors was not examined, it seems reasonable to assume that they were internalized, as previously described in neutrophils and HL60 cells (22–25). The second process accompanying desensitization is an uncoupling of formyl peptide receptors from their G proteins. Following desensitization, the remaining surface receptors failed to interact with G proteins, as determined by the inability of GTPγS and GDP to regulate receptor affinity and by the inability of FMLP to stimulate GTPγS binding and GTPase activity in DM.

The relative contribution of receptor loss and receptor-G protein uncoupling to desensitization was not addressed in our studies. The failure of desensitized cells to respond to FMLP with a respiratory burst despite the continued presence of some formyl peptide receptors, however, suggests that receptor-G protein uncoupling contributes to desensitization. The mechanism by which membrane formyl peptide receptors are uncoupled from G proteins remains to be determined. Both functional changes in the receptor-G protein interaction and physical sequestration of the receptor have been suggested as mechanisms for uncoupling (reviewed in Ref. 1). Previous reports indicate that the ligand-formyl peptide receptor complex interacts with the cytoskeleton, rendering the receptor incapable of stimulating a cellular response despite continued binding of the ligand (26, 39). These studies suggest that physical sequestration of formyl peptide receptors within the membranes may contribute to desensitization. Our finding that formyl peptide receptors on cell membranes isolated from the cytoskeleton are unable to interact with their G proteins, however, supports the view that a functional alteration in the formyl peptide receptor uncouples the receptor-G protein interaction, independent of any receptor sequestration.

Although our data are consistent with the hypothesis that desensitization alters the receptor-G protein interaction, other possible explanations for these findings include a reduction of FMLP receptors or a reduction of available G protein. The number of receptors in DM was reduced by about 75%, while the affinities of the receptor populations were not altered. We examined the role of disparate numbers of receptors in the failure of DM to interact with G proteins by including different amounts of membrane protein in the GTPγS binding assay. By adding DM in an amount 4 times that of NM, the total number of formyl peptide receptors in each assay should have been approximately equal. Despite the presence of the same number of receptors, FMLP stimulated GTPγS binding in NM but failed to stimulate GTPγS binding in DM. Because guanine nucleotides also failed to regulate FMLP binding in DM, it seems unlikely that the difference in receptor numbers could account for the failure to detect receptor-G protein interaction in DM.

The structural and functional integrity of membrane G proteins was examined by several methods. First, the ability of NaF to elicit O_2^- release in desensitized cells demonstrates a normal functional G protein component. Second, immunoblots using an antibody to a C terminal decapeptide of the α subunit of transducin in [^{32}P]ADP ribosylation catalyzed by pertussis toxin demonstrated insignificant differences in the quantities of G proteins between DM and NM. Although it is possible that a decrease in the relevant G protein was offset by an increase in another pertussis toxin-sensitive G protein, the alterations in G protein activation by the formyl peptide receptor in DM do not appear to result from quantitative differences in G proteins. Thus, we believe that our data provide evidence that a functional receptor-G protein uncoupling accompanies formyl peptide receptor desensitization.

Wilde *et al.* (40) recently reported similar findings in rabbit neutrophils. They demonstrated a time-dependent decrease in receptor number and in the efficiency with which formyl peptide receptors stimulated GTPase activity during desensitization. The loss of receptor efficiency of G protein activation was independent of the loss of membrane receptors. No quantitative or qualitative alterations in membrane G proteins were detected. They concluded that receptor-G protein uncoupling and down-regulation of receptors contribute to the loss of responsiveness during desensitization.

Studies in our laboratory have recently shown that Mg^{2+} regulates the interaction of the formyl peptide receptor with its G protein (reviewed in Ref. 41). Increasing concentrations of Mg^{2+} were shown to increase the proportion of receptors that demonstrate high affinity, to enhance the potency of GTPγS to inhibit FMLP binding, and to enhance the potency of FMLP to stimulate GTPase activity in membranes from normal HL60 cells. Therefore, the ability of Mg^{2+} to regulate receptor-G protein interactions in DM was examined. Our results show that Mg^{2+} is able to alter receptor affinity, to induce receptor sensitivity to guanine nucleotides, and to permit FMLP stimulation of GTPγS binding and GTPase activity in DM. Thus, Mg^{2+} not only regulates receptor affinity in DM but is able to partially restore G protein interaction with the receptor. Although this restoration was incomplete, it cannot be determined from these studies whether this was a consequence of reduced receptor numbers. These results suggest that uncoupling is a partially reversible process. The ability of Mg^{2+} to regulate desensitized receptor-G protein coupling may provide a useful tool for examining the mechanisms of desensitization.

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References

1. Sibley, D. R., and R. J. Lefkowitz. Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature (Lond.)* **317**:124–129 (1985).
2. Harden, T. K. Agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.* **35**:5–32 (1983).
3. Hertel, C., and J. P. Perkins. Review: receptor-specific mechanisms of desensitization of β-adrenergic receptor function. *Mol. Cell. Endocrinol.* **37**:245–256 (1984).
4. Stadel, J. M., P. Nambi, R. G. L. Shorr, D. F. Sawyer, M. G. Caron, and R. J. Lefkowitz. Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **80**:3173–3177 (1983).
5. Benovic, J. L., R. H. Strasser, M. G. Caron, and R. J. Lefkowitz. β-Adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. USA* **83**:2797–2801 (1986).
6. Benovic, J. L., C. Staniszewski, F. Mayor, Jr., M. G. Caron, and R. J. Lefkowitz. β-Adrenergic receptor kinase. *J. Biol. Chem.* **263**:3893–3897 (1988).
7. Su, Y., T. K. Harden, and J. P. Perkins. Catecholamine-specific desensitization of adenylate cyclase. *J. Biol. Chem.* **255**:7410–7419 (1980).
8. Stadel, J. M., B. Strulovici, P. Nambi, T. N. Lavin, M. M. Briggs, M. G. Caron, and R. J. Lefkowitz. Desensitization of the β-adrenergic receptor of frog erythrocytes. *J. Biol. Chem.* **258**:3032–3038 (1983).
9. Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. Characterization of an altered membrane form of the β-adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* **258**:13900–13908 (1983).
10. Chang, D., and E. Costa. Evidence for internalization of the recognition site

of β-adrenergic receptors during receptor subsensitivity induced by (−)-isoproterenol. *Proc. Natl. Acad. Sci. USA* **76**:3024–3028 (1979).
11. Strulovici, B., J. M. Stadel, and R. J. Lefkowitz. Functional integrity of desensitized β-adrenergic receptors. *J. Biol. Chem.* **258**:6410–6414 (1983).
12. Strasser, R. H., and R. J. Lefkowitz. Homologous desensitization of β-adrenergic receptor-coupled adenylate cyclase. *J. Biol. Chem.* **260**:4561–4564 (1985).
13. Kassis, S., and P. H. Fishman. Functional alteration of the β-adrenergic receptor during desensitization of mammalian adenylate cyclase by β-agonists. *Proc. Natl. Acad. Sci. USA* **81**:6686–6690 (1984).
14. Iyengar, R., M. K. Bhat, M. E. Riser, and L. Birnbaumer. Receptor-specific desensitization of the S49 lymphoma cell adenylyl cyclase. *J. Biol. Chem.* **256**:4810–4815 (1981).
15. Strasser, R. H., G. L. Stiles, and R. J. Lefkowitz. Translocation and uncoupling of the β-adrenergic receptor in rat lung after catecholamine promoted desensitization *in vivo*. *Endocrinology* **115**:1392–1400 (1984).
16. Toews, M. L., G. L. Waldo, T. K. Harden, and J. P. Perkins. Relationship between an altered membrane form and a low affinity form of the β-adrenergic receptor occurring during catecholamine-induced desensitization. *J. Biol. Chem.* **259**:11844–11850 (1984).
17. Bouvier, M., W. P. Hausdorff, A. De Blasi, F. B. O'Dowd, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz. Removal of phosphorylation sites form the β_2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature (Lond.)* **333**:370–373 (1988).
18. Wilden, U., S. W. Hall, and H. Kuhn. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc. Natl. Acad. Sci. USA* **83**:1174–1178 (1986).
19. Kuhn, H., and U. Wilden. Deactivation of photoactivated rhodopsin by rhodopsin-kinase and arrestin. *J. Recept. Res.* **7**:283–289 (1987).
20. Snyderman, R., C. D. Smith, and M. W. Verghese. Model for leukocyte regulation by chemoattractant receptors: roles of a guanine nucleotide regulatory protein and polyphosphoinositide metabolism. *J. Leuk. Biol.* **40**:785–800 (1986).
21. Gierschik, P., D. Sidiropoulos, C. Stannek, M. Steislinger, E. Herrmann, C. Straub, R. Moghtader, and K. H. Jakobs. The role of guanine nucleotide binding proteins in regulation of transmembrane signalling in neutrophils, in *Hormones & Cell Regulation* (J. Nunez and J. E. Dumont, eds.) Vol. 11. John Libbey Eurotext Ltd., Paris, 79–94 (1987).
22. Simchowitz, L., J. P. Atkinson, and I. Spilberg. Stimulus-specific deactivation of chemotactic factor-induced cyclic AMP response and superoxide generation by human neutrophils. *J. Clin. Invest.* **66**:736–747 (1980).
23. Anderson, R., and J. Niedel. Processing of the formyl peptide by HL-60 cells. *J. Biol. Chem.* **259**:13309–13315 (1948).
24. Sklar, L. A., D. A. Finney, Z. G. Oades, A. J. Jesaitis, R. G. Painter, and C. G. Cochrane. The dynamic of ligand-receptor interactions. *J. Biol. Chem.* **259**:5661–5669 (1984).
25. Jesaitis, A. J., J. R. Naemura, R. G. Painter, L. A. Sklar, and C. G. Cochrane. The fate of an *N*-formylated chemotactic peptide in stimulated human granulocytes. *J. Biol. Chem.* **258**:1968–1977 (1983).
26. Vitkauskas, G., H. J. Showell, and E. L. Becker. Specific binding of synthetic chemotactic peptides to rabbit peritoneal neutrophils: effects on dissociability of bound peptide, receptor activity and subsequent biologic responsiveness (deactivation). *Mol. Immunol.* **17**:171–180 (1980).
27. Jesaitis, A. J., J. O. Tolley, and R. A. Allen. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. *J. Biol. Chem.* **261**:13662–13669 (1986).
28. Johnson, R. A., and T. F. Walsh. The enzymatic preparation of [α-^{32}P]ATP, [α-^{32}P]GTP, [^{32}P]cAMP, and [^{32}P]cGMP, and their use in the assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res.* **10**:135–168 (1979).
29. Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **75**:2669–2673 (1978).
30. Aktories, K., G. Schultz, and K. H. Jakobs. Islet-activating protein prevents nicotinic acid-induced GTPase stimulation and GTP but not GTPγS-induced adenylate cyclase inhibition in rat adipocytes. *FEBS Lett.* **156**:88–92 (1983).
31. Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA* **75**:2458–2462 (1978).
32. Gierschik, P., D. Sidiropoulos, A. Spiegel, and K. H. Jakobs. Purification and immunochemical characterization of the major pertussis-toxin-sensitive guanine-nucleotide-binding protein of bovine-neutrophil membranes. *Eur. J. Biochem.* **165**:185–194 (1987).
33. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
34. Rodbard, D. H., H. Lenox, H. L. Way, and D. Ramseth. Statistical characterization of the random errors in the radioimmunoassay dose-response variable. *Clin. Chem.* **22**:350–358 (1976).
35. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
36. Aktories, K., G. Schultz, and K. H. Jakobs. Stimulation of a low K_m GTPase by inhibitors of adipocyte adenylate cyclase. *Mol. Pharmacol.* **21**:336–342 (1982).
37. Gierschik, P., J. Falloon, G. Milligan, M. Pines, J. I. Gallin, and A. Spiegel. Immunochemical evidence for a novel pertussis toxin substrate in human neutrophils. *J. Biol. Chem.* **261**:8058–8062 (1986).
38. Koo, C., R. J. Lefkowitz, and R. Snyderman. Guanine nucleotides modulate the binding affinity of the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. *J. Clin. Invest.* **72**:748–753 (1983).
39. Jesaitis, A. J., J. O. Tolley, R. G. Painter, L. A. Sklar, and C. G. Cochrane. Membrane-cytoskeleton interactions and the regulation of chemotactic peptide-induced activation of human granulocytes: the effects of dihydrocytochalasin B. *J. Cell. Biochem.* **27**:241–253 (1985).
40. Wilde, M. W., K. E. Carlson, D. R. Manning, and S. H. Zigmond. Chemoattractant-stimulated GTPase activity is decreased on membranes from polymorphonuclear leukocytes incubated in chemoattractant. *J. Biol Chem.* **264**:190–196 (1989).
41. Gierschik, P., K. McLeish, and K. H. Jakobs. Regulation of G protein-mediated signal transfer by ions. *J. Cardiovasc. Pharmacol. 12* **(Suppl. 5)**:S20–S24 (1988).

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