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Receptor-mediated ADP-ribosylation of a phospholipase C-stimulating G protein

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In membranes of myeloid differentiated HL 60 cells, the chemotactic peptide FMLP stimulates phospholipase C via a pertussis toxin-sensitive G protein. FMLP markedly stimulates the cholera toxin-dependent ADP-ribosylation of a 40 kDa protein in these membranes. This effect of FMLP is inhibited by GTP and GTP[S], and is almost completely abolished in membranes of pertussis toxin-pretreated HL 60 cells. Treatment of HL 60 membranes with cholera toxin and NAD markedly inhibits FMLP-stimulated high affinity GTPase. These results suggest that a 40 kDa G protein sensitive to both pertussis and cholera toxin functionally interacts with the formyl peptide receptor of HL 60 cells and, thus, very likely is the G protein that stimulates phospholipase C in this system.

G protein; ADP-ribosylation; Cholera toxin; Formyl peptide; Neutrophil; Phospholipase C

1. INTRODUCTION

Recent evidence suggests that guanine nucleotide-binding proteins are involved in coupling a variety of calcium mobilizing receptors to phospholipase C [1]. In certain cell types, e.g. human leukemia (HL 60) cells, neutrophils, mast cells and fibroblasts, the relevant G protein is functionally inactivated by pertussis toxin. In these systems, pertussis toxin ADP-ribosylates a 40 kDa membrane protein that is structurally different from described pertussis toxin substrates [2]. While this protein has been suggested to be the G protein that stimulates phospholipase C, direct evidence for coupling of the protein to phospholipase C or to calcium mobilizing receptors, has so far not been presented.

Membranes prepared from myeloid differentiated HL 60 cells contain high numbers of formyl peptide receptors which are coupled to stimulation of phospholipase C [3] and do not interact with the adenylate cyclase system [4,5]. Therefore, this sytem provides an excellent model to study structure and function of G proteins involved in coupling receptors to phospholipase C. In the present study we used a novel approach, i.e. receptor-mediated ADP-ribosylation of a G protein, to demonstrate, without relying on reconstitution techniques, that a 40 kDa G protein is functionally coupled to the formyl peptide receptor in native HL 60 membranes. Our results strongly suggest that this protein is the G protein that couples formyl peptide receptors to phospholipase C in HL 60 membranes.

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Abbreviations: G protein, guanine nucleotide-binding protein; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GTP[S], guanosine-5′-*O*-(3-thiotriphosphate); GppNHp, guanylyl-imidodiphosphate

2. MATERIALS AND METHODS

HL 60 cells were grown in suspension culture and induced to differentiate into mature myeloid

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forms by cultivation in the presence of 1.25% (v/v) dimethyl sulfoxide (DMSO) for 5 days [6]. Membranes were prepared as described [2] except that centrifugation through Percoll gradients was omitted.

For [^{32}P]ADP-ribosylation of membrane proteins, cholera toxin (2 mg/ml, Sigma) was activated by dilution with an equal volume of 40 mM dithiothreitol and incubated for 10 min at 30°C. Membranes (50–100 μg protein) were incubated for 60 min at 37°C in a volume of 50 μl containing 100 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 cholera toxin, 1 μM [^{32}P]NAD (20 μCi/nmol), and guanine nucleotides and FMLP at concentrations indicated in the figure legends.

For treatment of membrane proteins with cholera toxin and non-radioactive NAD, membranes (1 mg/ml) were incubated for the times indicated in a buffer containing 50 mM triethanolamine/HCl, pH 7.4, 2 mM ATP, 10 mM NAD, 2.5 mM $MgCl_2$, and 100 μg/ml of activated cholera toxin or an equal volume of the carrier solution. At the end of the incubation period, the membranes were washed at 4°C with 10 mM triethanolamine/HCl, pH 7.4, and directly used for determination of GTPase activities.

Hydrolysis of [γ-^{32}P]GTP (0.1 μCi per tube) was determined in a reaction mixture (100 μl) containing 5–10 μg membrane protein, 50 mM triethanolamine/HCl, pH 7.4, 1 mM dithiothreitol, 0.1 mM EGTA, 2 mM $MgCl_2$, 100 mM NaCl, 0.1 μM GTP, 0.1 mM ATP, 5 mM creatine phosphate (sodium salt), 0.4 mg/ml creatine kinase, and 0.2% (w/v) bovine serum albumin. Reactions were performed for 10 min at 25°C in the absence or presence of 1 μM FMLP. Reactions were terminated and high affinity GTPase activity was determined as described [7].

[γ-^{32}P]GTP and [^{32}P]NAD were synthesized as described [8,9]. SDS-polyacrylamide gel electrophoresis and autoradiography were performed as described in [2]. Protein was determined according to Bradford [10] using bovine IgG as a standard.

3. RESULTS AND DISCUSSION

When HL 60 membranes were treated with cholera toxin and [^{32}P]NAD, addition of the chemotactic peptide FMLP to the reaction mixture markedly stimulated the ADP-ribosylation of a 40 kDa membrane protein (fig.1). In contrast, there was no effect of FMLP on the cholera toxin-dependent ADP-ribosylation of the 43 kDa substrate which presumably corresponds to the α-subunit of the stimulatory G protein of adenylate cyclase, G_s. The effect of FMLP was concentration-dependent with half-maximal and maximal effects at approx. 0.1 and 1 μM, respectively. FMLP had no effect on the labelling of proteins in the absence of cholera toxin (not shown).

Fig.2 shows the effects of guanine nucleotides on the cholera toxin-dependent ADP-ribosylation of the 40 kDa membrane protein. As described before [11,12], in the absence of guanine nucleotides there was significant ADP-ribosylation of the 40 kDa substrate even in the absence of FMLP. This basal labelling was reduced or even absent in the presence of GTP and the metabolically stable GTP-analogs GppNHp and GTP[S]. In contrast, only GTP[S] and GTP, but not GppNHp, inhibited the effect of FMLP on labelling of the 40 kDa protein. This inhibition suggests that the stimulation of ADP-ribosylation of the 40 kDa protein by FMLP cannot be attributed to the stimulation of FMLP-dependent effector enzymes, e.g. phospholipase C or protein kinase C, since these effects of FMLP are enhanced or even mimicked by GTP and GTP[S] [13,14].

Effects of guanine nucleotides on ADP-ribosylation of membrane proteins by bacterial toxins do not necessarily imply that the substrate itself is a GTP-binding protein. A second G protein, also referred to as ADP-ribosylation factor (ARF) has recently been shown to be required for ADP-ribosylation of G_s by cholera toxin [15]. However, binding of GTP[S] or GTP to ARF stimulates, rather than inhibits, the ADP-ribosylation of G_s [15]. Hence, it appears unlikely that GTP[S] and GTP act via a G protein similar or identical to ARF. Instead, the findings indicate that a complex of the active formyl peptide receptor with the nucleotide-free 40 kDa protein serves as the actual cholera toxin substrate, and that binding of both GTP[S] and GTP to the 40 kDa substrate inhibits the formation of this complex.

At first glance, the striking difference between the two metabolically stable GTP analogues,

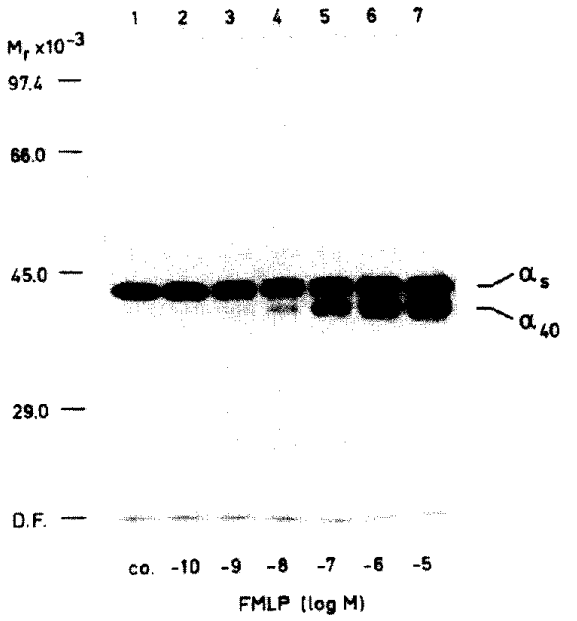

Fig.1. Stimulation by FMLP of the cholera toxin-dependent ADP-ribosylation of a 40 kDa protein in HL 60 membranes. Membranes were incubated with cholera toxin, [^{32}P]NAD and the indicated concentrations of FMLP. As FMLP is maximally effective in supporting the labelling of the 40 kDa protein the presence of GppNHp (see fig.2), 100 μM GppNHp, was included in the reaction mixture. The samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography of the dried gel was performed. The 40 kDa cholera toxin substrate comigrates on SDS-polyacrylamide gels with the 40 kDa pertussis toxin substrate of HL 60 membranes. The 43 kDa cholera toxin substrate comigrates with the α-subunits of G_s, which are present in membranes prepared from wild type S 49 lymphoma cells, but absent from cyc$^-$ mutant membranes [29] (not shown).

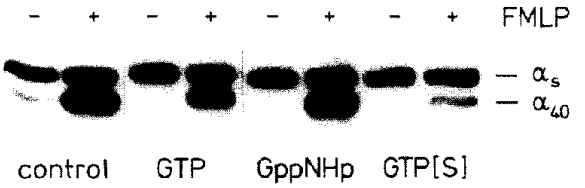

Fig.2. Effect of guanine nucleotides on the cholera toxin-dependent ADP-ribosylation of the 40 kDa substrate. HL 60 membranes were incubated with cholera toxin and [^{32}P]NAD in the absence (control) and presence of 100 μM of the indicated guanine nucleotides and in the absence (−) or presence (+) of 10 μM FMLP. The samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography of the dried gel was performed. Only the 40 kDa region of the autoradiogram is shown.

GTP[S] and GppNHp, in affecting the FMLP-stimulated ADP-ribosylation of the 40 kDa protein may appear to be contradictory. However, a similar difference has been reported for the cholera toxin-dependent ADP-ribosylation of transducin, which is also stimulated by an activated receptor, i.e. light-activated rhodopsin [16,17]. In this system, GppNHp was found to actually stimulate the G protein ADP-ribosylation, whereas GTP[S] was strongly inhibitory. Thus, the GppNHp-, but not the GTP[S]-ligand form, of both transducin and the HL 60 substrate appears to be capable of interacting with activated receptors and subsequently serving as a substrate for the toxin. In fact, a similar, fundamental difference between GppNHp and GTP[S] in affecting receptor-G protein coupling has also been observed for the coupling of G_s to the β-adrenoceptor [18].

The ADP-ribosylation of the 40 kDa G protein by cholera toxin is functionally correlated with a marked inhibition of FMLP-stimulated GTPase activity (fig.3). Half-maximal and maximal (80%) inhibition was seen after preincubation of the membranes with the toxin for 15 and 120 min, respectively. This time dependence strongly suggests that cholera toxin inhibits FMLP-stimulated GTP hydrolysis by an enzymatic activity and is consistent with an ADP-ribosylation of a G protein being the mechanism of action of the toxin. The inhibition of FMLP-stimulated GTPase activity by cholera toxin cannot be attributed to a toxin-induced increase in cAMP formation, since the addition of 100 μM cAMP and 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine to the preincubation mixture, to the buffer used for determination of GTP hydrolysis, or both, had no effect on GTP hydrolysis both in the absence and presence of FMLP (not shown). Cholera toxin-dependent ADP-ribosylation of G_s results in an inhibition of the GTPase activity of G_s [19]. It appears unlikely, however, that ADP-ribosylation of G_s leads to an inhibition of the FMLP-dependent GTPase activity, since there is no evidence that formyl peptide receptors couple

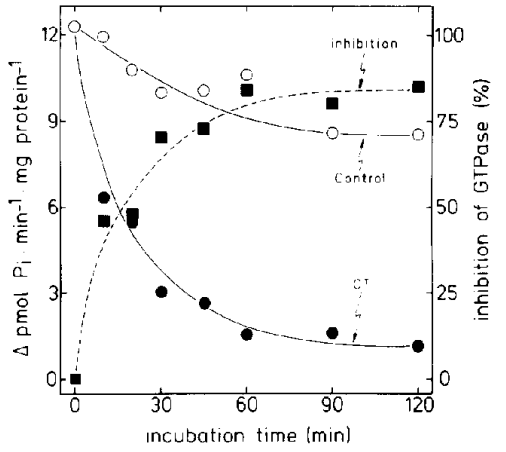

Fig.3. Inhibition by cholera toxin of the FMLP-stimulated GTPase activity in HL 60 membranes. HL 60 membranes were treated with (●) or without (○) activated cholera toxin (CT). At the indicated time points, membranes were withdrawn and the FMLP-stimulated GTPase activity was determined. Given at the ordinate is the increase in high affinity GTPase activity caused by the addition of 1 μM FMLP, which was about 2.2-fold at $t = 0$. The inhibitory effect of cholera toxin on FMLP-stimulated GTPase activity is strictly dependent on the presence of NAD during the preincubation, and is dependent on the concentration of the toxin. Half-maximal and maximal effects were seen at concentrations of about 5 and 100 μg/ml (not shown).

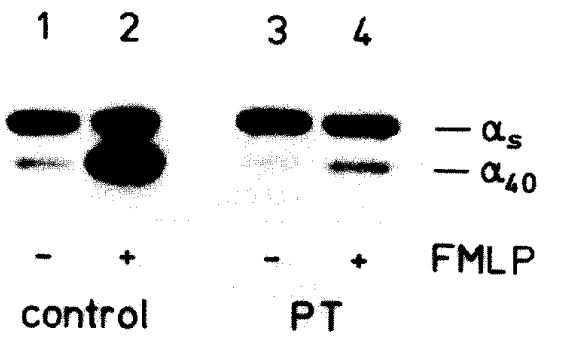

Fig.4. Inhibition by pertussis toxin of the FMLP-stimulated ADP-ribosylation of the 40 kDa substrate. HL 60 cells were treated for 3 h at 37°C with (lanes 3 and 4) or without (lanes 1 and 2) pertussis toxin (PT, 500 ng/ml). Membranes were prepared and treated with [^{32}P]NAD and activated cholera toxin in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 10 μM FMLP. The samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography of the dried gel was performed. Only the 40 kDa region of the autoradiogram is shown.

to G_s in HL 60 membranes. Similarly unlikely is an interaction of formyl peptide receptors with transducin, since several polyclonal antisera strongly reactive against transducin fail to detect any crossreactive material in HL 60 membranes. Thus, the results suggest that a G protein distinct from G_s and transducin, most likely the 40 kDa substrate, gives rise to the cholera toxin-sensitive GTPase activity in response to FMLP.

There is the distinct possibility that the cholera toxin-dependent ADP-ribosylation of the G protein coupled to the formyl peptide receptor also inhibits the stimulation of FMLP-dependent effector enzymes, including phospholipase C. Inhibition of several neutrophil functions has been noticed by several investigators [20–24]. Specifically, the inhibition of macrophage chemotaxis by cholera toxin has been reported to be independent of a toxin-dependent increase in cellular cAMP [11]. In addition, in at least two other systems, there is a marked, cAMP-independent inhibition by cholera toxin of phosphoinositide hydrolysis [25,26].

ADP-ribosylation of G proteins by pertussis toxin generally leads to a functional uncoupling of the modified G protein from the corresponding receptor. When intact HL 60 cells are treated with pertussis toxin, and membranes were tested for ADP-ribosylation of proteins by cholera toxin, there was a significant inhibition of basal and an almost complete loss of FMLP-stimulated ADP-ribosylation of the 40 kDa protein (fig.4). These findings strongly suggest that a single 40 kDa protein serves as a substrate for both toxins in HL 60 membranes. The major 40 kDa pertussis toxin substrate of neutrophils and HL 60 cells is structurally different from all previously described pertussis toxin substrates [2]. Therefore, our data suggest that this novel GTP-binding protein, which we have named G_n, is a substrate for cholera toxin when bound to the formyl peptide receptor. This demonstration of a functional interaction with a calcium mobilizing receptor provides strong evidence that this G protein is involved in regulation of phospholipase C in this system.

It is presently unknown whether a single amino acid or two different sites of the 40 kDa substrate

are modified by cholera and pertussis toxin. There is good evidence, however, that two different amino acids, cysteine-346 and arginine-174, are ADP-ribosylated by pertussis and cholera toxin, respectively, on the α-subunit of transducin [27,28]. As the 40 kDa protein of HL 60 membranes is very similar to transducin in terms of the characteristics and functional consequences of the toxin-dependent modifications, the 40 kDa protein may be ADP-ribosylated at two distinct sites as well. Interestingly, the primary structure derived from all cDNA clones coding for signal transducing G proteins isolated to date contain an arginine in a position that corresponds to arginine-174 of α_t [29]. Therefore, other G proteins, e.g. the inhibitory G protein of adenylate cyclase (G_i) or G_o, a G protein of unknown function found in brain, may also undergo agonist-stimulated ADP-ribosylation by cholera toxin. If true, this would provide a powerful tool to determine, within the native plasma membrane, which G protein couples to a given receptor, e.g. inhibitory receptors of adenylate cyclase, or to identify the receptors that interact with a specific G protein.

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