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DIFFERENTIAL STIMULATION OF THE GTPase ACTIVITY OF G-PROTEINS BY POLYLYSINE

MARCELO ANTONELLI,* JUAN OLATE,* ROLF GRAF,†‡ CATHERINE C. ALLENDE§ and JORGE E. ALLENDE*‖

*Departamento de Bioquímica, Facultad de Medicina, and §Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago 7, Chile; and †Department of Medicine, Baylor College of Medicine, Houston, TX, U.S.A.

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Abstract—Polylysine, polyornithine and, to a lesser extent, polyarginine were found to stimulate the GTPase activity of the purified recombinant α subunit of the human G_{i-3} transducing protein α_{i-3}. Optimal stimulation of 4- to 5-fold was obtained with polysine concentrations between 1 and 20 μM, higher concentrations being inhibitory. Polylysine at similar concentrations stimulated by 50% the GTPase of transducin (G_T), the vision transducing protein, but had only a very slight effect on the GTPase of the p21 product of the H-*ras* protooncogene. The stimulation of the α_{i-3} GTPase caused by polylysine was due to a reduction of the apparent K_m for GTP from 3.8 to 1.3 μM. The stimulation by polylysine was observed at free Mg^{2+} concentrations below 1 μM. These results indicate that polylysine acts in a fashion similar to mastoparan and substance P in mimicking the action of an agonist-bound receptor on G-proteins.

The transduction of many extracellular signals involves heterotrimeric proteins that bind GTP and GDP and have intrinsic GTPase activity. This family of analogous proteins is commonly known as G-proteins [for reviews see Refs. 1–3].

It has been established that agonist-bound receptors act on G-proteins by enhancing the exchange of GDP for GTP, favoring the formation of the complex of the α subunit of a specific G-protein with the nucleotide triphosphate. The α subunit–GTP complex, in turn, interacts with an effector molecule and either stimulates or inhibits its activity. The intrinsic GTPase activity of the α subunit hydrolyzes the bound nucleotide triphosphate causing the disengagement from the effector protein and returning the G-protein to its basal form that contains bound GDP.

Some progress has been made in our knowledge of the structural features that are responsible for the interactions between the agonist-bound receptor and the G-protein. Experiments in which cloned receptor genes have been modified through the formation of chimerae with other receptor genes or through site-directed mutagenesis have demonstrated that the third intra-cellular loop (loop i3) of the receptor protein is mainly responsible for their capacity to interact with their specific G-protein and to favor guanine nucleotide exchange [4–6]. It has been pointed out that this receptor loop can form amphipathic helices in which the polar side contains several basic amino acid residues [4]. Highly pertinent to these observations have been the findings of Higashijima *et al.* [7, 8], who have studied in great detail the effect of mastoparan, a tetradecapeptide rich in lysine that has the capacity to mimick the action of the ligand-bound receptor on several G-proteins. Mastoparan also can form an amphipathic helix with peptides containing polycationic clusters. Some synthetic peptides with sequences analogous to mastoparan or to the receptor loops also have been tested for their effect on G-protein binding of guanine nucleotides or on their GTPase activity [9, 10].

In a similar fashion, studies with mutants of the genes coding for α subunits of G-proteins have pointed to the involvement of the C-terminal portion of this subunit as participating directly in its interaction with the receptor [11, 12]. However, cross-linking analyses using mastoparan also point to the N-portion of some G-proteins as important for receptor analogue interaction [8].

Previous work from our laboratory has shown that polylysine and other polyamines have a stimulating effect on several interesting membrane enzymes such as adenylyl cyclase, phosphatidylinositol kinase and protein kinases [13]. More recently we have investigated further the action of polylysine on the membrane-bound adenylyl cyclase found in *Xenopus laevis* oocytes [14]. This study demonstrated that polylysine activates the adenylyl cyclase through the stimulatory G-protein (G_s) and that this polycation acts on this enzyme through a mechanism similar to that of the receptor in that it increases the binding of the guanosine triphosphate and increases the activity at low Mg^{2+} concentrations. In the present paper, experimental evidence is reported to demonstrate that polylysine can stimulate the GTPase activity of an isolated α subunit of human G_{i-3} and to a lesser extent of the trimeric transducin (G_T) molecule. Polylysine had very little effect on the GTPase activity of the p21 product of the H-*ras* protooncogene.

‡ Present address: Institute of Zoology, University at Zürich, Ch-8057 Zurich, Switzerland.

‖ Corresponding author: Dr. Jorge E. Allende, Casilla 70086, Santiago 7, Chile. Tel. and FAX (56-2) 376320.

MATERIALS AND METHODS

Materials. Poly-L-lysine (average M_r 10,200), poly-L-ornithine (average M_r 25,000) and poly-L-arginine (average M_r 40,000) were purchased from the Sigma Chemical Co. [γ^{32}P]GTP was synthesized in our laboratory using the method of Walseth and Johnson [15].

GTPase assay. The standard assay measures the appearance of extractable inorganic phosphate as phosphomolybdic acid by the method described by Martin and Doty [16] and also used by Conway and Lipimann [17] to measure the GTPase activity of protein synthesis factors. Incubations were carried out for 6 min at 30° in 100-μL mixtures containing: 15 mM Tris–HCl, pH 8.0; 5 mM $MgCl_2$; 20 mM β-mercaptoethanol; 1.1 mM EDTA; 50 μg/mL of bovine serum albumin (BSA); 1 μM [γ^{32}P]GTP (sp. act. of approximately 3000 cpm/pmol); a variable amount of GTPase enzyme; and, where indicated, polylysine or another polycationic peptide. The reaction was stopped by adding 200 μL of a 0.02 M solution of silicotungstic acid in 0.02 NH_2SO_4. Subsequently, 100 μL of 5 mM KH_2PO_4 was added as carrier to each tube, followed by 200 μL of 5% (w/v) ammonium molybdate in 4 M NH_2SO_4. The phosphomolybdate formed was extracted with 600 μL of a 1:1 mixture of benzene and isobutyl alcohol, mixing for 15 sec in a vortex and spinning for 1 min in an Eppendorf centrifuge to separate the organic and water phases. An aliquot of 300 μL of the organic phase was counted in a scintillation counter to estimate the amount of radioactive free inorganic phosphate formed by the GTPase activity.

The α subunit of human liver $G_{i\text{-}3}$ protein was produced from recombinant baculovirus expressed in Sf9 cells [18]. Recombinants were detected by hybridization analysis of DNA from infected cells, isolated by successive plaque purification rounds, and the identity was confirmed by the presence of the recombinant sequence in the cells infected with purified virus. Homogenates obtained from infected cells, where $G_{\alpha i\text{-}3}$ represented about 15% of the total protein, were centrifuged and $G_{\alpha i\text{-}3}$ was subsequently purified by sequential FPLC over DEAE-Sephacel, Mono-Q, Mono-S, and hydroxyapatite columns. The purified protein was analyzed by polyacrylamide gel electrophoresis and two protein bands were observed. The major band (70%) was microsequenced and corresponded to the conserved $G_{\alpha i\text{-}3}$ sequence. Microsequencing of the minor band indicated a similar structure resulting from initiation of translation at the second methionine residue [3].

The p21 protein product of the H-*ras* protooncogene of mice was synthesized in *Escherichia coli* from a plasmid expression vector and purified as described [19].

RESULTS

Effect of different polycationic peptides on the GTPase activity of the α subunit of $G_{i\text{-}3}$. The GTPase activity of the human liver $G_{i\text{-}3}$ α subunit ($\alpha_{i\text{-}3}$) produced from a cloned gene in a baculovirus expression vector was measured in the presence of polylysine, polyornithine and polyarginine. The results presented in Fig. 1 demonstrate that polylysine and polyornithine stimulated 4 to 5-fold the hydrolytic activity at concentrations of the polycation between 1 and 20 μM. Polyarginine was considerably less effective in stimulating the GTPase and completely inhibited the reaction at 20 μM.

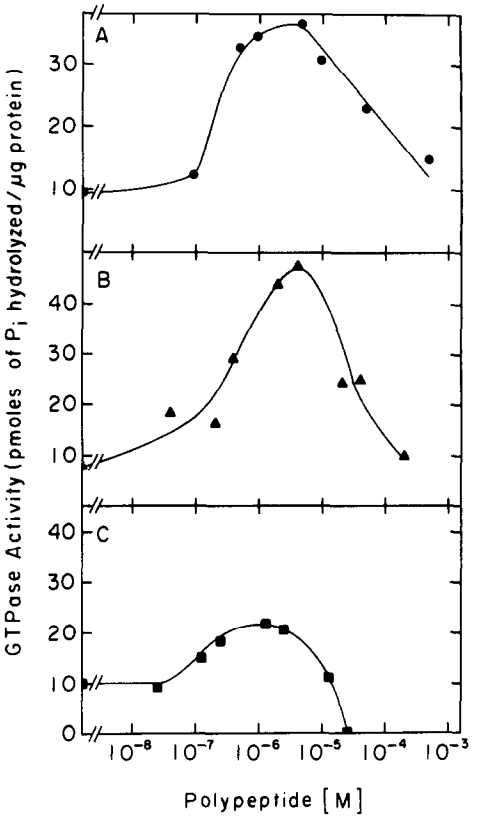

Fig. 1. Effect of the basic polypeptides on the GTPase activity of the $\alpha_{i\text{-}3}$ subunit. The GTPase activity of 250 ng of recombinant bovine $\alpha_{i\text{-}3}$ subunit was assayed as described in Materials and Methods except that the reaction was incubated for 20 min at 30°. In panel A, polylysine was added at the concentrations specified and the specific activity of the [γ-^{32}P]GTP was 6500 cpm/pmol. In panel B, polyornithine was added at the concentrations specified and the specific activity of the [γ-^{32}P]GTP was 2100 cpm/pmol. In panel C, polyarginine was added at the concentrations specified and the specific activity of the [γ-^{32}P[GTP was 1800 cpm/pmol. All points are averages of duplicate determinations.

Figure 2A shows the stimulatory effect of 20 μM polylysine at different concentrations of the $\alpha_{i\text{-}3}$ protein and Fig. 2B shows the time course of the reaction in the presence and absence of polylysine. These results establish that the stimulation occurs over a 10-fold range of concentrations of the enzyme and throughout the time course of the hydrolytic reaction.

Effect of polylysine on other GTP binding proteins with GTPase activity. Studies were carried out to determine whether polylysine could also stimulate the GTPase activity of other GTP binding proteins.

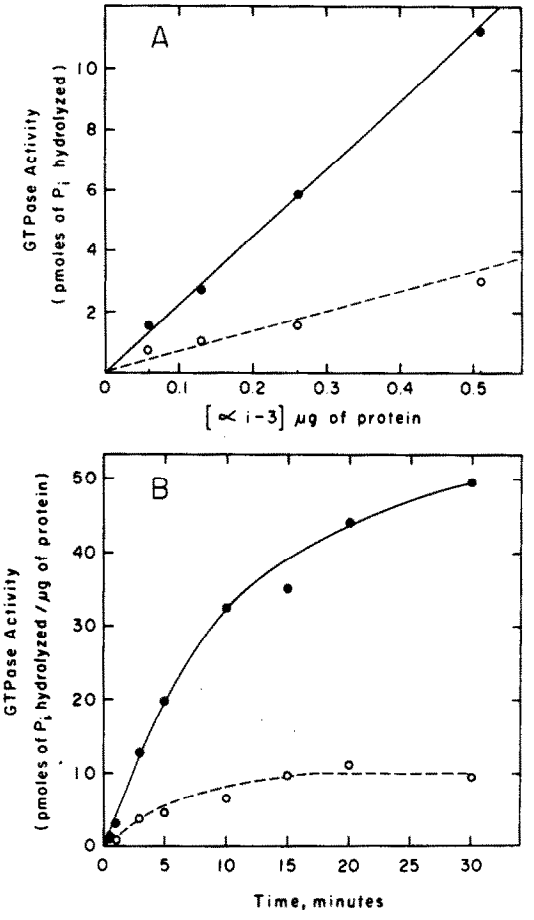

Fig. 2. Effect of different $\alpha_{i\text{-}3}$ enzyme concentrations and of different times of incubation on the GTPase activity measured in the presence and absence of polylysine. The GTPase activity of the recombinant $\alpha_{i\text{-}3}$ subunit was measured essentially as described in Materials and Methods, and the values given are averages of duplicate determinations. In panel A, the reaction was incubated for 6 min at 30° using the specified amounts of $\alpha_{i\text{-}3}$ and 1 μM [γ-^{32}P]GTP with a specific activity of 6000 cpm/pmol in the presence (●) or absence (○) of 20 μM polylysine. In panel B, the incubations contained 250 ng of $\alpha_{i\text{-}3}$ and 1 μM [γ-^{32}P]-GTP with a specific activity of 5600 cpm/pmol and were carried out in the presence (●) or absence (○) of 20 μM polylysine.

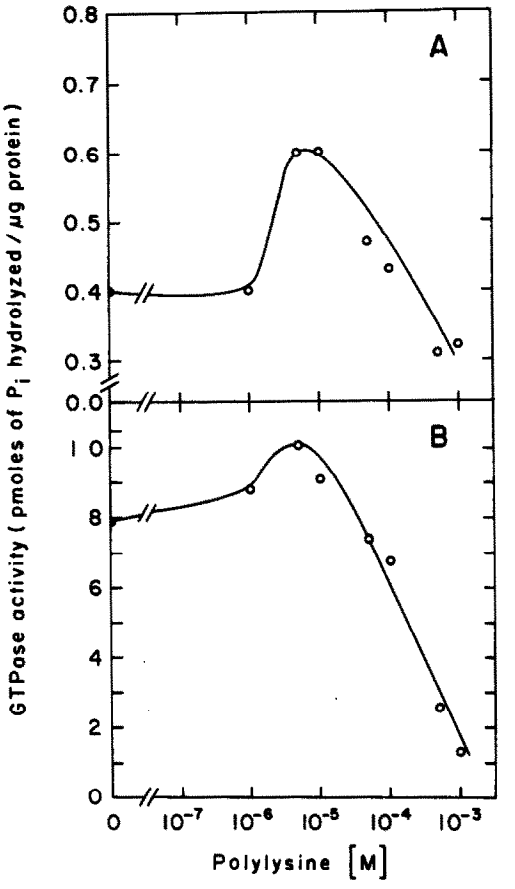

Fig. 3. Effect of polylysine on the GTPase activity of transducin and the p21 product of H-*ras* protooncogene. The GTPase was measured essentially as described in Materials and Methods. The results are representative of two experiments, and points are averages of duplicate determinations. In panel A, 6.8 μg of pure bovine transducin was used with 1 μM [γ-^{32}P]GTP of specific activity 1330 cpm/pmol. The reactions were incubated for 20 min at 30°, and the concentration of $MgCl_2$ added was 1.1 mM. In panel B, 1.6 μg of the p21 product of the H-*ras* protooncogene was used with 1 μM [γ-^{32}P]GTP of a specific activity of 5500 cpm/pmol. Incubations were for 40 min at 30°.

Figure 3A shows the effect of polylysine on the GTPase activity of transducin (G_T), a trimeric G-protein involved in vision [20]. Concentrations of polylysine of 10 μM stimulated the GTPase activity of G_T approximately 50%. It must be pointed out that the specific activity of the GTPase of G_T was more than one order of magnitude lower than that observed with $\alpha_{i\text{-}3}$.

Figure 3B shows the effect of polylysine on the GTPase activity of the p21 product of the H-*ras* protooncogene produced in *E. coli*. There was only a slight stimulation observed around 5 μM polylysine. Polylysine at concentrations of 100 μM was clearly inhibitory to the GTPase of the H-*ras* p21.

Effect of polylysine on the apparent K_m *for GTP of* $\alpha_{i\text{-}3}$ *and on the* Mg^{2+} *requirement of the reaction.* The velocity of the GTPase reaction catalyzed by $\alpha_{i\text{-}3}$ was tested at different concentrations of GTP in the presence and absence of a stimulatory amount of polylysine (Fig. 4). The results obtained demonstrate that the presence of polylysine permitted an increase in the affinity of the enzyme for GTP. The Eadie–Hofstee graph of these results, shown in the insert of Fig. 4, demonstrates that the apparent K_m for GTP was decreased by polylysine from 3.8 μM to approximately 1.3 μM, while a similar V_{max} was reached.

The results shown in Fig. 5 present the effect of different concentrations of Mg^{2+} on the GTPase reaction of $\alpha_{i\text{-}3}$ in the presence and absence of polylysine. It is clear that polylysine stimulated the

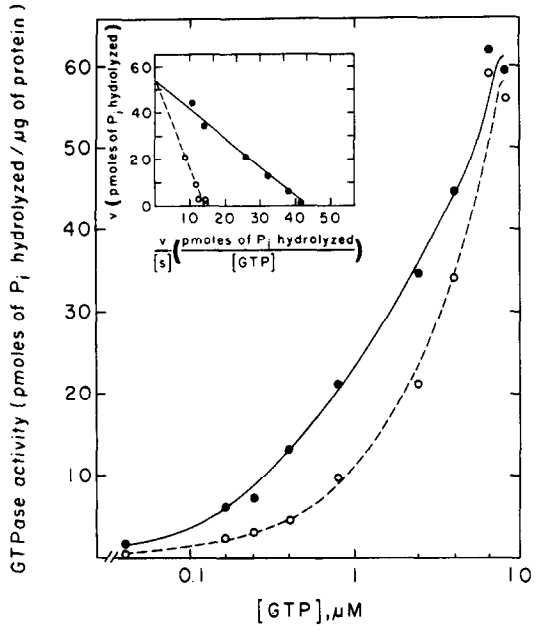

Fig. 4. Effect of GTP concentration on the GTPase activity of the $\alpha_{i\text{-}3}$ subunit. The GTPase activity was measured essentially as described in Materials and Methods. The results are representative of three experiments, and points are averages of duplicate determinations. The reaction mixtures contained 230 ng of $\alpha_{i\text{-}3}$ and different concentrations of [γ-^{32}P]GTP. The incubations were for 6 min at 30° and were carried out in the presence (●) or absence (○) of 20 μM polylysine. The inset shows an Eadie–Hofstee graph of the same data.

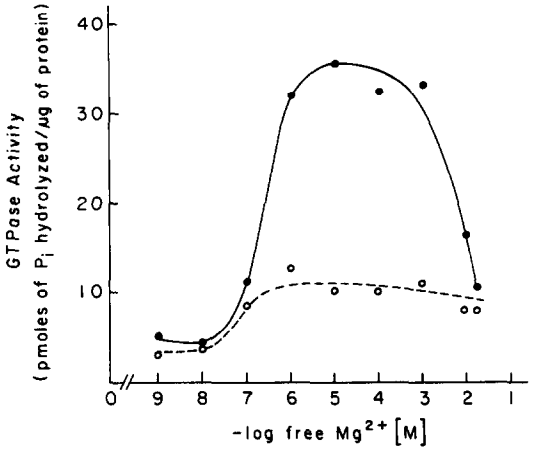

Fig. 5. Effect of free Mg^{2+} concentration on the GTPase activity of the $\alpha_{i\text{-}3}$ subunit measured in the presence and absence of polylysine. The GTPase activity was measured essentially as described in Materials and Methods. The incubations were for 20 min at 30°, contained 200 ng of $\alpha_{i\text{-}3}$ and 1 μM [γ-^{32}P]GTP with 5200 cpm/pmol, and were carried out in the presence (●) or absence (○) of 20 μM polylysine and the specified concentration of free Mg^{2+}. The free concentration of Mg^{2+} was calculated by a computer program prepared by Storer and Cornish-Bowden [21]. The points are averages of duplicate determinations.

reaction in a range of Mg^{2+} from 10^{-6} to 10^{-2} M. No stimulation was observed at the two extremes of the metal ion concentration and no significant shift in the Mg^{2+} requirement seemed to be caused by polylysine.

DISCUSSION

The results presented demonstrate that polylysine and polyornithine, and to a lesser extent polyarginine, can strongly stimulate the GTPase activity of some G-proteins. The clearest results were obtained with the human $\alpha_{i\text{-}3}$ subunit which had been expressed from a cloned gene in a baculovirus system and extensively purified. The concentrations of the polycationic peptides required to yield a 4- to 5-fold stimulation of its GTPase were in the micromolar range, a concentration which is more than one order of magnitude lower than the optimal concentration of polylysine required to stimulate the membrane-bound adenylate cyclase of *X. laevis* [14]. These concentrations of polylysine are also lower than the concentrations of mastoparan required for the activation of the GTPase activity of G_o [7]. It is also interesting that concentrations of the polycationic homopolymers above 50 μM were clearly inhibitory, a phenomenon which is not observed with mastoparan.

The capacity of polylysine to stimulate the GTPase activity is not uniform for all proteins that possess this function. The polylysine stimulation of the GTPase activity of transducin was clear but was much lower than that found with $\alpha_{i\text{-}3}$. In this respect it is also important to note that the basal GTPase specific activity of transducin was more than one order of magnitude lower than that of $\alpha_{i\text{-}3}$. A previous report [22] indicated that the hydrolytic activity of transducin is very low and it increases markedly upon its reconstitution into membranes containing rhodopsin. It is also suggested that a conformational change in transducin is necessary for GTPase activity [22]. It is important to note that polylysine does not stimulate significantly the hydrolysis of GTP by the p21 *ras* protein. This difference in response to polylysine is not surprising since it has been found that various different GAP proteins [23] activate the GTPase activity of the *ras* gene products. There does not appear to be any structural relationship between these GAP proteins and the membrane receptors that interact with the trimeric G-proteins.

The polylysine stimulation of the GTPase activity observed in this study with $\alpha_{i\text{-}3}$ seems to be due to a lowering of the apparent K_m for GTP. A similar effect was observed in our studies on the effect of polylysine on the activation of adenylyl cyclase mediated by G_s [14].

The effect of polylysine on the Mg^{2+} curve of the GTPase reaction of $\alpha_{i\text{-}3}$ demonstrated that micromolar concentrations of the metal are sufficient to effect the stimulation by polylysine. Not surprisingly, the Mg^{2+} curve obtained for the GTPase reaction differed from the results which we reported for the much more complex system of the membrane-bound adenylyl cyclase measured with or without polylysine [14]. In the case of the membrane-bound adenylyl cyclase, a second rise in the activity is

observed at millimolar concentrations of Mg^{2+}. It is known that in this system there is multiple involvement of Mg^{2+}. On the other hand, the bell-shaped Mg^{2+} curve obtained with polylysine and the GTPase of $\alpha_{i\text{-}3}$ is very similar to that reported by Higashijima *et al.* [7] for the Mg^{2+} effect on the GTPase of G_o stimulated by mastoparan.

The results reported here demonstrate that polylysine and other polycationic peptides can act on G-proteins particularly the α subunits of trimeric G-proteins to affect their interaction with guanine nucleotides at micromolar Mg^{2+} concentrations. In this action they are similar to mastoporan and related peptides such as substance P that mimic the agonist-bound receptors. These findings support the proposal that basic amino acids are of importance in the interaction of receptors with G-proteins. However, these results indicate that amphipathic helices or specific sequences are not essential requirements for the effect that polycationic polymers have on interactions of G-proteins with guanine nucleotides.

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