Mechanisms for Inhibition of the Catalytic Activity of Adenylate Cyclase by the Guanine Nucleotide-binding Proteins Serving as the Substrate of Islet-activating Protein, Pertussis Toxin* (Received for publication, September 17, 1985)

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Two GTP-binding trimeric proteins (referred to as $\alpha_i\beta\gamma$ and $\alpha_p\beta\gamma$ based on the kilodalton molecular weights of their $\alpha$-subunits) were purified from rat brain as the specific substrates of the ADP-ribosylation reaction catalyzed by islet-activating protein, pertussis toxin, and resolved irreversibly into $\alpha$- and $\beta\gamma$-subunits by incubation with guanosine 5'-O-(thiotriphosphate) (GTP$\gamma$S). Some of these resolved subunits interacted directly with the adenylate cyclase catalyst partially purified from rat brain in a detergent-containing solution, resulting in inhibition of the cyclase activity as follows. 1) GTP$\gamma$S-bound $\alpha_i$: inhibited the catalyst, but GTP$\gamma$S-bound $\alpha_p$ did not; the inhibition was competitive with GTP$\gamma$S-bound $\alpha$-subunit of N$_i$, the GTP-binding protein involved in activation of adenylate cyclase. 2) $\beta\gamma$ from either $\alpha_i\beta\gamma$ or $\alpha_p\beta\gamma$ inhibited the catalyst in a manner not competitive with the activator such as forskolin or the $\alpha$-subunit of N$_i$. 3) The ADP-ribosylation of $\alpha_i\beta\gamma$ by islet-activating protein did not exert any influence on the subsequent GTP$\gamma$S-induced resolution and the ability of the resolved GTP$\gamma$S-bound $\alpha_i$ to inhibit the catalyst. 4) The $\beta\gamma$-induced inhibition of the catalyst was additive to the inhibition caused by GTP$\gamma$S-bound $\alpha_i$. Thus, the direct inhibition of the catalyst by $\beta\gamma$ or GTP$\gamma$S-bound $\alpha_i$ is a likely mechanism involved in receptor-mediated inhibition of adenylate cyclase, in addition to the previously proposed indirect inhibition due to the reduction of the concentration of the active $\alpha$-subunit of N$_i$, by reassociation with $\beta\gamma$.

Recent studies have revealed that the hormone-sensitive adenylate cyclase system consists of at least three types of proteins, cell surface receptors for a number of hormones and neurotransmitters, the stimulatory or inhibitory guanine nucleotide-binding regulatory component (N$_i$ or N$_o$), and the catalytic protein (see Ref. 1 for review). N$_i$ has a heterotrimeric structure with 45,000-, 35,000-, and -10,000-Da subunits (2-4). N$_o$ has a similar subunit structure with 41,000-Da (y) subunits of N$_i$ and N$_o$ were indistinguishable from each other in their functions (13) or by analysis of amino acid compositions and maps of proteolytic peptides (14). The idea is currently accepted that membrane-bound N$_o$, to mediate receptor-coupled activation of the adenylate cyclase catalyst, continuously traverses a kinetic regulatory cycle (15) in which: (a) extracellular signals via membrane receptors stimulate the binding of GTP to the $\alpha$-subunit of N$_i$ (16, 17) and the concomitant subunit dissociation into $\alpha$- and $\beta\gamma$-subunits (see Ref. 1 for review); (b) the GTP-bound $\alpha$-subunit of N$_i$ thus formed activates directly the catalyst of adenylate cyclase (2); and (c) the bound GTP is hydrolyzed to GDP due to GTPase activity of the $\alpha$-subunit (16, 18, 19) leading to deactivation of this peptide which is probably accompanied by reassociation with $\beta\gamma$. In the case of N$_i$, too, receptor-mediated increases in GTP$\gamma$S binding (4, 5) and GTPase (6, 7, 20) activity were observed in phospholipid vesicles into which receptors and N$_i$ had been reconstituted (21, 22). The purified N$_o$ was likewise dissociated into $\alpha$- and $\beta\gamma$-subunits upon addition of GTP$\gamma$S (13, 24, 25). Thus, a similar kinetic cycle is applicable to N$_o$-induced inhibition of adenylate cyclase, though the exact roles of $\alpha$- and $\beta\gamma$-subunits resolved from N$_o$ in inhibition of the catalytic part remain unclear as yet.

The $\beta\gamma$-subunits resolved from N$_i$ (2, 26) and N$_o$ (13, 24, 25) were capable of association with the $\alpha$-subunit of N$_i$ thereby deactivating membrane adenylate cyclase. Thus, the decrease in the $\alpha$-subunit of N$_i$ due to its reassociation with $\beta\gamma$-subunits arising from N$_i$ is one of the proposed mechanisms by which N$_i$ inhibits adenylate cyclase (13, 24, 25). An additional and exceptional mechanism so far proposed postulates direct interaction of the $\alpha$-subunit of N$_i$ with the catalyst to explain the inhibition of adenylate cyclase in S49 cyc-$^-$ cell membranes in which N$_i$ is lacking (25, 27, 28). The purpose of the present communication is to report for the first time the data showing direct interaction of the $\alpha$- and $\beta\gamma$-subunits resolved from N$_i$ with the partially purified catalyst of adenylate cyclase in a detergent-containing solution.

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1 The abbreviations used are: N$_i$ and N$_o$, the guanine nucleotide regulatory components of adenylate cyclase that mediate stimulation and inhibition, respectively (they have also been referred to as G$_i$ and G$_o$); IAP, islet-activating protein (pertussis toxin); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; GTP$\gamma$S, guanosine 5'-O-(thiotriphosphate); Chaps, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; SDS, sodium dodecyl sulfate.


Both α- and β-γ-subunits interacted with the catalyst at different sites leading to inhibition of adenylate cyclase activity.

**EXPERIMENTAL PROCEDURES**

**Purification of N, and IAP Substrates and Resolution of Their Constituent Subunits—**N, was purified from rabbit liver membranes as described previously (4). The procedures employed to purify two IAP substrate proteins (α4β and αγβ) from rat brain membranes were based on the methods developed for purification of N, from rabbit liver (4) and will be described elsewhere. Resolution of the IAP substrates to GTPγS-bound α- and γ-β-subunits was performed by high-performance gel filtration. Two-hundred µl of αβγ or αγβ (~500 µg) which had been incubated for 2 h at 30 °C with HED (50 mM sodium Hepes (pH 8.0), 0.1 mM sodium EDTA, 0.1 mM dithiothreitol), 0.1% Lubrol, 25 mM MgCl₂ containing 50 µM [35S]GTPγS (~500 cpm/pmol) was applied to a TSK-3000SW column (0.75 x 80 cm), which had been equilibrated with TED (20 mM Tris-HCl (pH 8.0), 0.1 mM sodium EDTA, 1 mM dithiothreitol), 0.6% Chaps, 100 mM Na₂SO₄, 10 mM MgSO₄. Elution was then achieved with the same buffer with the flow rate of 0.5 ml/min and the fraction volume of 0.25 ml. Fig. 1 details the activities and absorbance at 280 nm of the IAP substrates or N, was quantitated by SDS-polyacrylamide gel (12%) electrophoresis, and the silver stain of proteins is shown in Figs. 1A and 2. The preparations used in the present study were more than 95% pure. GTPγS-treated N, was prepared by high-performance gel filtration. Two-hundred µl of samples eluted from columns was added to an assay mixture (90 µl); the composition (with the final concentrations) of the resultant mixture was 50 mM sodium Hepes (pH 8.0), 1 mM TEDTA, 3 mM MgCl₂, 2.5 µM [α32P]ATP (~500 cpm/pmol), 3 mM phosphoenolpyruvate, 10 µg/ml pyruvate kinase, 0.2 mM 4-(3-hydroxy-4-methoxy benzyl)-2-imidazoline, 0.1 mg/ml bovine serum albumin, and 10 µM forskolin. After incubation for 15 min at 30 °C, the reaction was terminated, and [32P]cAMP was isolated and counted by the method of Salomon et al. (29).

For assay of the adenylate cyclase activity of the final step preparation of catalyst, as influenced by N proteins, 10 µl of the preparation of catalyt (10 µg of protein in TED, 0.1% Lubrol, 100 mM NaCl) was mixed with 20 µl of αβγ (or αγβ) or its resolved subunits (in TED, 0.6% Chaps, 100 mM Na₂SO₄, 10 mM MgSO₄) and maintained on ice for 15 min. The samples were further incubated for 30 min at 30 °C with 70 µl of the above assay mixture containing 5 mM MgCl₂ instead of MnCl₂ and lacking forskolin. The [32P]cAMP was assayed as above.

**RESULTS**

Resolution of the IAP Substrates to GTPγS-bound α- and γ-β-Subunits—Two IAP substrates purified from rat brain are used in experiments in this communication. One is N, first identified as the selective IAP substrate with an α-subunit of 41,000 Da (4, 31, 32) and henceforth referred to as ααβγ, while the other is an additional IAP substrate with a 39,000 Da α-subunit which is probably the same as αβγ termed by Sternwise and Robishaw (5) and referred to as αβγ. Incubation of either ααβγ or αβγ with GTPγS resulted in dissociation into GTPγS-bound αγ, or GTPγS-bound αβ and γ which were successively separated from each other by high-performance liquid chromatography (Fig. 1). Just the chromatographic patterns as those in Fig. 1 were obtained when experiments were repeated under the same conditions with ααβγ or αβγ that had been ADP-ribosylated with IAP and NAD (data not shown).

The fractions eluted from the chromatographic column were assayed for their effects on the adenylate cyclase activity of the isolated catalyst. The catalyst used was a preparation from rat brain membranes which was, though still crude, essentially devoid of N, ααβγ, αγβ, or their constituent subunits (see Table I). The fractions containing γγ were strongly inhibitory to the catalyst. The inhibition was observed even in the absence of N, in the assay mixture, indicating that γγ was capable of directly interacting with the catalyst. In the case of ααβγ, the profile of this inhibitory activity displayed a shoulder corresponding to the first peak of GTPγS-bound α (Fig. 1A). No shoulder was observed unless the assay mixture were fortified with activated N. Nor was there any shoulder when αβγ was studied. It was anticipated, therefore, that N, induced activation of the adenyl cyclase catalyst was antagonized by GTPγS-bound αγ but not by GTPγS-bound αβ. The results in Fig. 1 then urged us to further study the direct effect of the resolved α- and β-γ-subunits on the catalyst as will be described below.

The resolved subunits of IAP substrates, together with ADP-ribosylated ones, were subjected to SDS-polyacrylamide gel electrophoresis.
Inhibition of Adenylate Cyclase Catalyst by IAP Substrates

Fig. 1. Resolution of the subunits of GTPγS-treated IAP substrates by high-performance gel filtration. Purified αβγ (Panel A) or αβδγ (Panel B) was incubated with [35S]GTPγS plus Mg2+, and the [35S]GTPγS-bound α was resolved from βγ by means of a TSK-3000SW column as described under “Experimental Procedures.” Aliquots (20 μl) of the preparations were assayed for their effect on the adenylate cyclase activity of partially purified catalyst in the presence (●) or absence (○) of GTPγS-treated N, (0.5 pmol) as described under “Experimental Procedures.” Activities are expressed as percentages of the control values obtained with the elution buffer alone, which were 180 and 35 pmol of cAMP formed/tube for plus and minus N, respectively. Additional aliquots (5 μl) were counted for [35S], and nmol of [35S]GTPγS bound/ml is shown (○). The absorbance at 280 nm of the eluted protein was also monitored (---). The polypeptide composition of the protein eluted was analyzed by SDS-polyacrylamide gel (12%) electrophoresis, and the silver stain of protein on the gel is illustrated on the top.

Table I

Comparison of activities between the starting extract of rat brain membranes and the final-step preparation of the adenylate cyclase catalyst

<table>
<thead>
<tr>
<th>Activities</th>
<th>The starting extract</th>
<th>The final-step preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate cyclase (pmol/min/mg)</td>
<td>Mg2+</td>
<td>Mg2+ and 10 μM GTPγS</td>
</tr>
<tr>
<td>Adenylate cyclase (pmol/min/mg)</td>
<td>29</td>
<td>520</td>
</tr>
</tbody>
</table>

*Aliquots (10 μl) of the preparations were assayed for adenylate cyclase activity with either 5 mM MgCl2 or 3 mM MnCl2 and indicated reagents as described under “Experimental Procedures.”

**Aliquots (10 μl) of the preparations were diluted 10-fold into HED, 0.1% Lubrol containing 25 mM MgCl2 and 10 μM GTPγS, and the N, activity was assayed as described previously.2

2 Aliquots (20 μl) of the preparations were assayed for IAP substrate activity as described previously.2

3 Aliquots (20 μl) of the preparations were mixed with 10 μl of HED, 0.1% Lubrol containing free αδ-subunit (10 pmol), and the mixture was [35P]ADP-ribosylated as described previously.4 The activity of βγ-subunits was estimated by the degree of ADP-ribosylation of α, since no significant substrate activity was detected when free α was submitted for ADP-ribosylation and the amount of ADP-ribosylation of α was dependent on the amount of βγ added.2

4 Not determined. The expected value was, however, more than IAP substrate activity, since the ADP-ribosylation of α required stoichiometric amount of βγ-subunits.2

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified subunits from two IAP substrates. Samples were prepared for electrophoresis as described previously.3 The electrophoresis was accomplished in a 12% polyacrylamide slab gel, and proteins were stained with silver. Individual lanes contained approximately 1 μg of: 1, GTPγS-bound αα; 2, GTPγS-bound αα, 3, βγ resolved from ααβγ (βγ2); 4, βγ resolved from ααβγ (βγα); 5, GTPγS-bound ADP-ribosylated αα; and 6, GTPγS-bound ADP-ribosylated αα.

Fig. 3. Effects of GTPγS-treated N, and forskolin on the activity of the adenylate cyclase catalyst. The partially purified catalyst of adenylate cyclase was incubated with indicated concentrations of GTPγS-treated N, in the presence (●) or absence (○) of 10 μM forskolin in Panel A, or incubated with indicated concentrations of forskolin in the presence (●) or absence (○) of 0.5 pmol of GTPγS-treated N, in Panel B. The adenylate cyclase activities were assayed as described under “Experimental Procedures.”

Activation of the Adenylate Cyclase Catalyst by N, and Forskolin—The activity of the adenylate cyclase catalyst was enhanced by the addition of forskolin to the assay mixture, as had been observed in the detergent-solubilized preparations of bovine brain membranes (33). N, purified from rabbit liver exhibited no stimulatory effect on the activity of the catalyst in the absence of guanine nucleotides (data not shown). Stimulation was observed when it had been treated with GTPγS (Fig. 3). Based on the previous results (2) that the dissociation of oligomeric N, to its activated monomeric state...
was facilitated by GTPγS or fluoride, it is very likely that this preincubation of N₆ with GTPγS afforded the active GTPγS-bound α₄₁-subunit of N₆. The activity of the catalyst increased progressively to a saturation level, when the concentration of GTPγS-treated N₆, (Fig. 3A) or forskolin (Fig. 3B) was increased.

Activations by forskolin and by GTPγS-treated N₆ were mutually additive; the concentration-response curve with respect to GTPγS-treated N₆ was shifted upward by the addition of a definite concentration of forskolin (Fig. 3A). Likewise, the degree of further activation induced by 0.5 pmol of GTPγS-treated N₆ was roughly the same at all the forskolin concentrations studied (Fig. 3B). It is very likely that the sites at which N₆ and forskolin interact with the catalyst are different from each other.

Inhibition of the adenylate cyclase catalyst by βγ and GTPγS-bound α₄₁—The inhibition of the adenylate cyclase catalyst by βγ-subunits of two IAP substrates as first observed in Fig. 1 was further investigated. The βγ-subunits were very potent inhibitors of the catalyst. The inhibition developed progressively in a hyperbolic fashion as the amount of βγ was increased (Fig. 4A). The concentration of βγ to cause the half-maximal inhibition was around 5 pmol/tube with two different amounts of the catalyst. As had been expected, the βγ inhibition was in the same degree regardless of whether the subunits originated from α₂βγ or from α₃βγ.

The inhibition by GTPγS-bound α₄₁-subunits was studied of the catalyst which was activated by GTPγS-treated N₆, since the fractions containing GTPγS-bound α₄₁ were inhibitory only when the assay mixture was fortified with the activated N₆ as observed in Fig. 1 (Fig. 4B). The GTPγS-bound α₄₁ inhibited the catalytic activity in a dose-dependent manner; the inhibition was still progressive beyond 80 pmol/assay tube of α₄₁, suggesting that the affinity of the catalyst for the α₄₁-subunit was rather low. No significant inhibition was, however, observed with GTPγS-bound α₀₀. Slight inhibition caused by high concentrations of α₀₀ would be explained by contamination of this preparation with a few per cent of either α₄₁, βγ, or both. An additional finding of interest was that GTPγS-bound α₄₁ was similarly inhibitory to the catalyst either before or after being ADP-ribosylated by IAP and NAD. The interaction of α₄₁ with the adenylate cyclase catalyst was not impaired by ADP-ribosylation of this GTP-binding protein.

Mode of Inhibition by βγ- and GTPγS-bound α₄₁—Inhibition of the adenylate cyclase catalyst by βγ was studied in the absence or presence of activators of the catalyst (Fig. 5). The βγ-induced inhibition was essentially the same on a percentage basis whether the base-line catalytic activity was increased or not by the addition of GTPγS-treated N₆ (Fig. 5, inset), confirming the results in Fig. 1. When 10 μM forskolin was included or Mg²⁺ was replaced by Mn²⁺ in the assay mixture, there was a marked increase in the catalytic activity attaining to the level comparable with that achieved by GTPγS-treated N₆. Addition of βγ to these activated states of catalyst caused marked inhibition, although the degree of inhibition was smaller than that observed with the N₆-activated catalyst. The half-maximal inhibition was around 5 pmol of βγ/tube under all the assay conditions tested (Fig. 5, inset). No competition was thus observed between βγ and these activators for the catalyst.

In contrast, the inhibition of the catalyst by GTPγS-bound α₄₁ was strictly dependent on the amount of GTPγS-treated N₆, simultaneously added (Fig. 6). No inhibition was observed unless GTPγS-treated N₆ was also included in the assay. The lack of inhibition was not due to the low activity of the catalyst in the absence of GTPγS-treated N₆, because the high activity obtained with forskolin was also resistant to GTPγS-bound α₄₁-induced inhibition. The Dixon plots (34) for GTPγS-bound α₄₁-induced inhibition were constructed at three different concentrations of GTPγS-treated N₆, (Fig. 6, inset). The plots at lower concentrations of GTPγS-bound α₄₁ were linear and intersected at a point in a left-hand quadrant, indicating that GTPγS-bound α₄₁ acted as a competitive inhibitor.
Inhibition of Adenylate Cyclase Catalyst by IAP Substrates

petitive inhibitor of GTPγS-bound α11 subunits (from N,) at these concentrations. Probably, α11 occupies the same sites on the catalyst proteins as does α11 (from N). At higher concentrations, however, there was a downward bend in each of the plots; rather complicated kinetics would be involved under these conditions (see "Discussion").

Combined effects of these two different inhibitors, the βγ- and GTPγS-bound α11 subunits, on the catalyst were studied as shown in Fig. 7. Even when the N,-stimulated activity of the catalyst was maintained at a fully low level by the addition of a large amount of βγ alone, the combined addition of increasing concentrations of GTPγS-bound α11 caused further progressive inhibition, suggesting that the inhibition site of GTPγS-bound α11 was independent from the site for βγ-induced inhibition. Again, βγ did, but GTPγS-bound α11 did not, inhibit the basal activity of the catalyst measured in the absence of GTPγS-treated N.

DISCUSSION

Incubation of N, N, (α11βγ), or another IAP substrate (α11βγ) with GTPγS, a nonhydrolyzable analogue of GTP, is a means to maintain these trimeric proteins in active states irreversibly, as a result of their dissociation into the GTPγS-bound α-components and the βγ-component which is common to all of these proteins. To our knowledge, the present communication is the first report of direct inhibition of the partially purified adenylate cyclase catalyst by the thus activated components of purified α11,βγ and α11βγ. Interaction of these protein components with the catalyst was allowed to proceed under certain "unphysiologic" conditions, i.e. in a detergent-containing solution. It is probable, however, that these proteins behave in qualitatively similar manners in cell membranes. The major findings obtained in the present communication are as follows.

βγ Inhibited the Catalyst as a Result of Their Direct Interaction—βγ proved to be a strong inhibitor of the adenylate cyclase catalyst (Figs. 4A, 5, and 7). The concentration of βγ required for the half maximal inhibition was ~5 pmol/tube (or ~50 pmol/ml) under all the experimental conditions studied. Characteristics of the βγ-induced inhibition are as follows. (a) Similar inhibition kinetics were observed either with nonactivated inhibitor or with the catalyst activated by GTPγS-treated N, suggesting that there was no cooperative interaction on the catalytic protein between sites for βγ inhibition and the GTPγS-bound α11 (from N,) activation. (b) The catalyst activated by Mn2+ or forskolin was less susceptible to the βγ-induced inhibition than was the catalyst activated by GTPγS-treated N,. Such was also the case with platelet membrane preparations (13). (c) The inhibition was not complete; the maximal inhibition induced by 80 pmol of βγ was around 55 and 40% of the control value for the catalyst activated with GTPγS-treated N and with forskolin, respectively. (d) The catalyst maximally inhibited by βγ was further inhibited by GTPγS-bound α11. Conceivably, α11 and βγ inhibited the cyclase by binding to different and independent sites on the catalytic protein.

α11 Inhibited the Catalyst Only When the Catalyst Was Activated by N,—The inhibition of the catalyst by GTPγS-bound α11 (Fig. 4B, 6, and 7) was in agreement with the previous findings that adenylate cyclase activity in human platelet (13) and S49 cye- cell (25) membranes was weakly lowered by the addition of purified GTPγS-bound α11. The inhibition was not observed unless the catalyst was simultaneously activated by GTPγS-treated N,. This indispensable role of GTPγS-treated N, was not mimicked by other activators, forskolin (Fig. 6) and Mn2+ (not shown). A likely explanation is that GTPγS-bound α11 antagonized the action of GTPγS-bound α11 (from N,) competitively by occupying the same sites on the catalytic protein. The Dixon plots (34) drawn with three different N, concentrations were all linear below 5 pmol/tube of α11 and intersected at a common point in a left-hand quadrant (Fig. 6, inset), indicating that GTPγS-bound α11 actually competed with α11 (from N,) for the activation sites at these lower concentrations. The inhibition constant (K,) given by the projection on the abcissa of the intersection point proved to be around 4 pmol/tube, which
Inhibition of Adenylate Cyclase Catalyst by IAP Substrates

Fig. 8. A proposed scheme for regulation of adenyl cyclase activity due to GTPγS-bound N, and N, by IAP, GTPγS-bound regulatory protein; Rs, receptor; a41, the 45,000-Da subunit of N, a41; N, the 41,000-Da subunit of N (a41b42γ); a41γ, the 39,000-Da subunit of a41b42γ; βγ, 35,000- and 30,000-Da subunits of N, N, and a41b42γ; Catalyst, the catalyst of adenyl cyclase; IAP, ilet-activating protein, pertussis toxin; a, stimulatory; i, inhibitory. See text for designation of sites.

was about 20 times as high as Kd obtained for the activation by GTPγS-treated N, alone under the same assay conditions (Fig. 3).

There was a downward bend on the Dixon plot when the concentration of GTPγS-bound a41 was increased beyond 5 pmol/tube. This might suggest cooperative interaction between multiple binding sites for this ligand at these higher concentrations. Alternatively, however, this apparently complex kinetics would merely reflect a contamination of the a41 preparation with a minute amount (less than 1%) of the a-subunit of N, which is capable of causing detectable activation at a concentration as low as 0.1 pmol/tube (Fig. 3A).

a39 Failed to Inhibit the Catalyst—GTPγS-bound a39, in sharp contrast with GTPγS-bound a41, did not inhibit the adenyl cyclase catalyst; a slight inhibition was explainable by a minute contamination of the preparation with a41 or βγ (Fig. 4B). a39b39γ from rat2 and bovine (5–7) brains behaved very similarly to a41b41γ from the same tissues when they were studied in a detergent solution for their GTPγS-binding and GTP-hydrolyzing activities as well as for their capability to be ADP-ribosylated by IAP. Both a41b41γ and a39b39γ were likewise coupled to purified2 or partially purified (35) muscarinic receptors in phospholipid vesicles or γ-aminobutyric acid B receptors in brain membranes (36). Thus, the ability of a39 to interact with the catalyst is its only property so far observed to differentiate it qualitatively from a41. Since βγ seems to be an important component responsible for adenyl cyclase inhibition (see below), a39b39γ must be as good a communicator as a41b41γ in receptor-linked inhibition of the cyclase catalyst in cell membranes. Nevertheless, it is tempting to speculate that a39 is capable of interaction with an enzyme other than adenyl cyclase. The candidate of the enzyme coupled to a39 is phospholipase C which has recently been reported to be linked to Ca2+-mobilizing receptors in an IAP-susceptible fashion in mast cells (37) and neutrophils (38–40). Receptor-mediated arachidonic acid release was also found to be abolished by prior exposure to IAP of mouse 3T3 fibroblasts (8) and NG108-15 hybrid cells (29); phospholipase A2 might be coupled to receptors via a39 in these cells. The validity of this speculation is currently under study in our laboratory.

ADP-ribosylation of a41 by IAP Did Not Affect a41-induced Inhibition of the Catalyst—Both a41 and a39 served as good substrates for IAP-catalyzed ADP-ribosylation; ADP-ribosylation occurred when the reaction was tightly bound to βγ in the absence of Mg2+.2 This ADP-ribosylation failed to affect the biological activities (such as GTPγS binding and GTP hydrolyzing) of the trimers and monomers which were measurable in a detergent solution. A difference has been observed so far between the ADP-ribosylated and native a41b41γ only when these proteins were reconstituted into phospholipid vesicles along with muscarinic (22) or GABA (36) receptors. The ADP-ribosylated N, was not coupled to these receptors. Taken together with the present finding that the inhibition of the catalyst by GTPγS-bound a41 was not affected by ADP-ribosylation, therefore, it is concluded that the capability of a41b41γ to couple to receptors was selectively impaired by ADP-ribosylation of the a-subunit.

Plausible Contribution of Three Inhibitory Mechanisms to Receptor-mediated Inhibition of Adenylate Cyclase in Membranes—Occupation of receptors with an agonist causes dissociation of receptor-coupled N, a41b41γ to GTP-bound a41 and βγ. It has been previously proposed that the thus formed βγ inhibits the cyclase activity rather indirectly by reducing the concentration of the free a-subunit of N, a direct activator of the catalyst, as a result of their association to form the inactive trimer of N (24, 25). Two additional mechanisms are now shown in the present communication for N-induced inhibition of cyclase; βγ inhibited catalytic activity as a result of its direct interaction with the catalyst, and GTP-bound a41, completed with the a-subunit of N, for the activation sites on the catalyst. These multiple mechanisms for inhibition of the adenyl cyclase catalyst are shown in the scheme in Fig. 8. The Kd values for binding to the catalyst were approximately 0.2 pmol for GTPγS-bound a-subunit of N, 4 pmol for GTPγS-bound a41, and 5 pmol for βγ/0.1 ml of detergent-containing solution. Such difference in the affinity would be overcome by a large excess of N, over N, in membranes in many cell types. Conceivably, all of the three inhibitory mechanisms could function in membranes when receptors (Ri) coupled to a41b41γ or a39b39γ are stimulated by agonists as illustrated in Fig. 8.

Adenyl cyclase activity was inhibited via N, even in the N,-deficient variant (cyt-) of S49 lymphoma (25, 27). The GTPγS-bound a41γ was inhibitory in cyt- cell membranes in the presence of forskolin (25). Thus, it still remains possible that cyt- cells contain a defective stimulatory subunit of N, with which GTP-bound a41γ might compete for the activation sites on the catalyst (25).

REFERENCES

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Inhibition of Adenylate Cyclase Catalyst by IAP Substrates