Adenylyl cyclase Regulates Signal Onset via the Inhibitory GTP-binding Protein, $G_i^*$

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Received for publication, February 29, 2000, and in revised form, June 12, 2000
Published, JBC Papers in Press, June 13, 2000, DOI 10.1074/jbc.M001687200

Adenylyl cyclase, the enzyme that converts ATP to cAMP, is regulated by its stimulatory and inhibitory GTP-binding proteins, $G_s$ and $G_i$, respectively. Recently, we demonstrated that besides catalyzing the synthesis of cAMP, type V adenylyl cyclase (ACV) can act as a GTP-activating protein for $G_s$ and also enhance the ability of activated receptors to stimulate GTP-GDP exchange on heterotrimeric $G_i$ (Scholich, K., Mullenix, J. B., Wittporth, C., Poppleton, H. M., Pierre, S. C., Lindorfer, M. A., Garrison, J. C., and Patel, T. B. (1999) Science 283, 1328–1331). This latter action of ACV would facilitate the rapid onset of signaling via $G_i$. Because the C1 region of ACV interacts with the inhibitory GTP-binding protein $G_i$, we investigated whether the receptor-mediated activation of heterotrimeric $G_i$ was also regulated by ACV and its subdomains. Our data show that ACV and its C1 domain increased the ability of a muscarinic receptor mimetic peptide (MIII-4) to enhance activation of heterotrimeric $G_i$ such that the amount of peptide required to stimulate $G_i$ in steady-state GTPase activity assays was 3–4 orders of magnitude less than without the C1 domain. Additionally, the MIII-4-mediated binding of guanosine 5′-($\gamma$-thio)triphosphate (GTPγS) to $G_i$ was also markedly increased in the presence of ACV or its C1 domain. In contrast, the C2 domain of ACV was not able to alter either the GTPase activity or the GTPγS binding to $G_i$ in the presence of MIII-4. Furthermore, in adenylyl cyclase assays employing S49 ccc− cell membranes, the C1 (but not the C2) domain of ACV enhanced the ability of peptide MIII-4 as well as endogenous somatostatin receptors to activate endogenous $G_i$ and to inhibit adenylyl cyclase activity. These data demonstrate that adenylyl cyclase and its C1 domain facilitate receptor-mediated activation of $G_i$.

Adenylyl cyclase (AC)$^1$, the enzyme that catalyzes the conversion of ATP to cAMP, is regulated by its stimulatory and inhibitory GTP-binding proteins, $G_s$ and $G_i$, respectively. The binding of hormones or neurotransmitters to their respective receptors that couple to either $G_s$ or $G_i$ results in the activation of these G proteins (reviewed in Refs. 1 and 2). Essentially, receptor-mediated activation of the heterotrimeric G proteins involves the exchange of GTP for GDP on the $\alpha$ subunit, and this results in the dissociation of the $\alpha$ from $\beta\gamma$ subunits (reviewed in Refs. 1 and 2). Although the activated $G_s$ subunit can stimulate the activity of all nine forms of membrane-bound adenylyl cyclases that have been cloned and characterized to date (reviewed in Ref. 1), activated $G_i$ inhibits only type V, VI, and I adenylyl cyclases (1, 3, 4).

Recently, we demonstrated that besides catalyzing the synthesis of cAMP, type V adenylyl cyclase (ACV) has two other functions. First, the enzyme can act as a GTP-activating protein for $G_s$ and thereby expedite the termination of signaling via activated monomeric $G_s$ (5). Second, adenylyl cyclase enhances the ability of activated β-adrenergic receptor mimetic peptide to stimulate GTP-GDP exchange on heterotrimeric $G_i$ (5). This latter action of adenylyl cyclase would facilitate the rapid onset of signaling. Interestingly, the regions on adenylyl cyclase that interact with $G_i$ (6) are sufficient to exert the GTPase-activating protein- and guanine nucleotide exchange-enhancing actions of the enzyme (5). Since we (3) and others (4) have shown that the C1 region of ACV interacts with the inhibitory GTP-binding protein $G_i$, we investigated whether the activation of heterotrimeric $G_i$ was also regulated by adenylyl cyclase and its subdomains. Our data demonstrate that adenylyl cyclase and its C1 domain increased the ability of an activated muscarinic receptor mimetic peptide to enhance activation of heterotrimeric $G_i$. These data and our previous results (5) demonstrate that adenylyl cyclase regulates receptor-mediated activation of both G proteins that modulate its activity.

EXPERIMENTAL PROCEDURES

Expression and Purification of C1-C2 ACV and Its Domains—The recombinant soluble C1-C2 ACV and its subdomains C1 and C2 were expressed in the TP2000 strain of Escherichia coli, which does not contain endogenous AC (7, 8). Expression of protein and cell lysis were performed as described previously (9), except that the induction with isopropyl-$\beta$-D-thiogalactopyranoside (0.1 mM) was performed at 23 °C for 15 h. The hexahistidyl-tagged proteins were then purified as described for C1-C2 ACV. Briefly, after cell lysis, the supernatant was batch-bound for 1 h at 4 °C to metal-chelating resin (Talon, CLONTECH), which was equilibrated in buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM $\beta$-mercaptoethanol. The resin was then poured into a column and washed with 10 column volumes of buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1 mM $\beta$-mercaptoethanol, followed by a second wash with 10 column volumes of buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM NaCl, and 1 mM $\beta$-mercaptoethanol. The proteins were eluted with 3 column volumes of 50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM $\beta$-mercaptoethanol, and 125 mM imidazole. The eluted proteins were then applied to a Mono Q 5/5 fast protein liquid chromatography column and eluted with a 30-mL gradient of NaCl (100–450 mM) in 50 mM Tris-HCl, pH 8.0. Fractions containing the proteins were pooled and concentrated in buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5% glycerol and stored at −80 °C.

Purification of G Protein $\alpha$ and $\beta\gamma$ Subunits—All of the G protein $\alpha$ subunits were expressed in the JM109(DE3) strain of E. coli as described by Lee et al. (10). $G_{i1}$ was coexpressed with yeast protein

* This work was supported by Grant HL59679 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: AC, adenylyl cyclase; ACV, type V adenylyl cyclase; DTT, dithiothreitol; GTPγS, guanosine 5′-($\gamma$-thio)triphosphate.
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N-myristoyltransferase to ensure synthesis of myristoylated G protein as described by Linder et al. (11). Purification of the recombinant myristoylated Ga1 subunit was achieved by the method of Mumby and Linder (12). For the purification of the hexahistidyl-tagged, constitutively active Q213L mutant of Ga1 (Ga1s*) (13), the harvested cells were lysed, with the conditions described by Lee et al. (10) (Ga1s* supernatant) as described above in the presence of 1 mM MgCl2.

RESULTS AND DISCUSSION

Several studies have shown that peptides corresponding to cytosolic domains of receptors can mimic the actions of activated receptors and can stimulate G proteins and subsequently the G protein effectors (5, 16, 18, 19). In this respect, these peptides can be regarded as constitutively active receptors. Okamoto and Nishimoto (19) have shown that a peptide corresponding to amino acids 382–400 (RNQVRKQRMA-RERKTR) in the third cytosolic loop of the M1 muscarinic cholinergic receptor (MIII-4) can efficiently activate Gi1. Therefore, in our studies, we employed peptide MIII-4 to activate heterotrimeric Gi1. The activation of heterotrimeric G proteins involves exchange of GTP for GDP on the α subunit. This GTP-GDP exchange is the rate-limiting step in the activation of G proteins and can be conveniently monitored by measuring the steady-state rate of GTP hydrolysis (5, 18, 20, 21).

Recently, we (3) and others (4) have demonstrated that Ga1G interacts with the C1 domain of ACV and inhibits enzyme activity. Therefore, in initial experiments, we investigated the ability of the C1 and C2 domains of ACV to modulate the steady-state GTPase activity of Gi1 in the presence of varying concentrations of peptide MIII-4. As demonstrated in Fig. 1A, increasing concentrations of peptide MIII-4 increased the steady-state GTPase activity of Gi1. However, in the presence of the C1 domain of ACV, the ability of the peptide to increase steady-state GTPase activity was markedly augmented such that the concentration-response curve was shifted to the left by ~3–4 log units. In control experiments, in the absence of peptide MIII-4, the C1 domain did not alter the steady-state GTPase activity of Gi1 (zero peptide concentration in Fig. 1A), and the C1 domain by itself also did not demonstrate any significant GTPase activity over background levels (data not shown). Moreover, the C2 domain of adenylyl cyclase did not alter the ability of peptide MIII-4 to modulate the steady-state GTPase activity of Gi1 (Fig. 1A). Similar to the C1 region, the C2 domain by itself also did not demonstrate any GTPase activity (data not shown). Therefore, these data show that the C1 domain of ACV, but not the C2 region of the enzyme, can augment the ability of the muscarinic receptor mimetic peptide MIII-4 to increase GTP-GDP exchange on Ga1.

It is now well established that interactions between the C1 and C2 domains of AC are required to reconstitute enzyme activity, which can be regulated by its modulators (3–5, 9). Therefore, to determine whether the C1 domain in the context of the adenylyl cyclase molecule can modulate the ability of MIII-4 to increase the steady-state GTPase activity of Ga1, the previously described (3, 5, 9), engineered, soluble form of ACV (C1-C2 ACV) was used. The data in Fig. 1B demonstrate that like the C1 domain, in the absence of MIII-4, C1-C2 ACV by itself did not alter the steady-state GTPase activity of Gi1. C1-C2 ACV by itself also did not demonstrate any significant GTPase activity over background levels (data not shown). However, similar to the data with the C1 domain, C1-C2 ACV markedly augmented the ability of peptide MIII-4 to stimulate GTPase activity (Fig. 1B). Thus, the GTP-GDP exchange-enhancing actions of the C1 domain of ACV are also observed when the C2 domain is present to interact with the C1 region.

Because the ability of threshold concentrations (0.1 μM) of MIII-4 to activate Gi1 could be markedly enhanced by the C1 domain and C1-C2 ACV (Fig. 1, A and B), in subsequent experiments, we employed MIII-4 at a concentration (0.1 μM) that by itself did not markedly stimulate the steady-state GTPase activity. Using this concentration of the peptide, we deter-
mined that the effects of C1-C2 ACV and its C1 domain to increase the GTPase activity of Gi was concentration-dependent and saturable (Fig. 1C). On the other hand, in control experiments, the C2 domain of ACV at concentrations up to 2.0 μM did not alter the ability of MIII-4 to activate Gi (Fig. 1C).

Activation of heterotrimeric G proteins also results in increased GTP binding to the Gα subunit (5, 16, 18). Therefore, as a second approach to monitoring Gi activation, we measured the binding of the non-hydrolyzable GTP analog GTPγS to Gi. These experiments were conducted under conditions (low Mg2+ and 100 nM GTPγS) that are optimized to observe peptide-induced increases in the ability of G proteins to bind GTPγS (5, 16, 18, 19). As demonstrated by the data in Fig. 2, neither the threshold concentrations of MIII-4 (0.1 μM) nor the addition of C1-C2 ACV or its subdomains (C1 and C2) in the absence of MIII-4 altered GTPγS binding to Gi. However, the presence of both MIII-4 and C1 or C1-C2 ACV increased GTPγS binding to the G protein (Fig. 2). Notably, the C2 domain of ACV, which did not modulate the ability of MIII-4 to stimulate the steady-state GTPase activity of Gi (Fig. 1A), also failed to increase GTPγS binding to Gi (Fig. 2). These data (Fig. 2) demonstrate that C1-C2 ACV and its C1 domain enhance the ability of MIII-4 to activate Gi and to increase GTPγS binding to the G protein.

To determine the functional significance of the ability of the C1 domain of ACV to augment the actions of MIII-4 for Gi activation, the experiments depicted in Fig. 3 were performed. Essentially, we monitored the ability of the C1 domain of ACV to modulate adenylyl cyclase activity via MIII-4-mediated activation of endogenous Gi in S49 cym- membranes. S49 cym- cells do not express Gαq (22) and therefore provide a convenient model to study Gi activation without interference from Gα activity. Moreover, S49 cym- cells express predominantly type VI AC (23), which is inhibited by Gαq (1, 2). In these experiments, the cym- membranes were incubated with or without peptide MIII-4 (0.1 μM) in the presence and absence of the C1 or C2 domain of ACV (each at 1.5 μM). AC activity assays were then performed in the presence of forskolin (33 μM). As demonstrated by the data in Fig. 3A, neither peptide MIII-4 (0.1 μM) nor the C1 domain of ACV altered AC activity in cym- membranes. However, when both MIII-4 and C1 were present together, the AC activity was inhibited by 50%. To determine if the inhibition of AC activity observed in the presence of MIII-4 and the C1 domain of ACV was due to activation of endogenous Gαq, similar experiments were performed in membranes of S49 cym- cells treated with pertussis toxin. Pertussis toxin ADP-ribosylates Gαq, and this modification of the G protein precludes its activation by receptors (24). As shown in Fig. 3A, in membranes from pertussis toxin-treated cells, the inhibition of AC activity in the presence of MIII-4 and the C1 domain was abolished. These data demonstrate that the inhibition of AC activity in the presence of MIII-4 and the C1 domain observed in membranes of S49 cym- cells not exposed to pertussis toxin is indeed due to activation of endogenous Gαq. Furthermore, in experiments with the C2 domain of ACV, AC activity was not affected even when the C2 domain was present along with MIII-4 (Fig. 3B). These data show that the C1 (but not the C2) domain of ACV enhances the ability of MIII-4 to activate endogenous Gαq in cym- cell membranes and to inhibit AC activity. The conclusion noted above was further substantiated by experiments in which AC activity in cym- cell membranes was stimulated with the constitutively active, GTPase-deficient mutant of Gαq (Q203L, Gαq*) (Fig. 4, A and B). Gαq* was bound to the non-hydrolyzable GTP analog GTPγS prior to experimentation (see “Experimental Procedures”). Therefore, changes in AC activity in cym- membranes cannot be attributed to alterations in the active form of Gαq*. As shown in Fig. 4A,
The means described for Gdemonstrated by the findings that when GTPa assays in the presence of G were used in adenylyl cyclase with and without the C1 domain (1.5 mM carbachol at concentrations up to 1 mM did not inhibit forskolin-stimulated adenylyl cyclase activity in cycm (0.1 mM), neither C1 nor threshold concentrations of peptide MIII-4 of the C1 domain. In cyc stimulated by G, was stimulated by G. Because Katada et al. (24) have shown that somatostatin via G activation inhibits adenylyl cyclase activity in cyc- cells, further experiments were performed with somatostatin. As shown in Fig. 5, in the absence of ACV subdomains, somatostatin inhibited forskolin-stimulated adenylyl cyclase activity in a concentration-dependent manner. Addition of the C2 domain of ACV did not alter the inhibition of adenylyl cyclase by somatostatin (Fig. 5). However, the C1 domain of ACV shifted the somatostatin concentration-response curve to the left so that the inhibition of enzyme activity at lower concentrations of somatostatin was enhanced in the presence of the C1 domain (Fig. 5). In control experiments, the C1 domain alone, in the absence of somatostatin, did not inhibit adenylyl cyclase activity (Figs. 5; also see Figs. 3 and 4). Moreover, in membranes of cyc- cells treated with pertussis toxin, neither somatostatin nor the combination of somatostatin and C1 domain altered adenylyl cyclase activity (data not shown). These data (Fig. 5) demonstrate that the paradigm that we have described employing a reconstituted G protein and a muscarinic receptor mimetic peptide (Figs. 1–4) is applicable to inhibition of adenylyl cyclase by endogenous G-coupled receptors. Thus, in the presence of the C1 region of ACV, lower amounts of agonist and therefore lower amounts of protein and a muscarinic receptor mimetic peptide (Figs. 1–4) are incubated in the absence or presence of peptide MIII-4 (0.1 mM) with or without the C1 domain as described under “Experimental Procedures.” These preincubated membranes were used in adenylyl cyclase with and without the C1 domain (1.5 mM carbachol at concentrations up to 1 mM) by themselves significantly altered AC activity that was stimulated by G or (Fig. 4A). However, the combination of MIII-4 and C1 inhibited AC activity by 60% (Fig. 4A). This effect was specific for the C1 domain of ACV since the C2 domain of ACV failed to alter AC activity in cyc- cells. That the inhibition observed in Fig. 4A was due to activation of endogenous Gi was demonstrated by the findings that when GTPγS was omitted from the preincubations of S49 cyc- membranes with or without MII-4 and/or the C1 domain, the inhibition shown in Fig. 4A was not observed (data not shown).

To determine if the C1 region of ACV could augment inhibition of adenylyl cyclase activity mediated by activation of endogenous receptors, experiments were performed with S49 cyc- cell membranes. To complement the experiments with the muscarinic receptor mimetic peptide MIII-4, the ability of the muscarinic receptor agonist carbachol to inhibit the activity of adenylyl cyclase in cyc- membranes was assessed. However, carbachol at concentrations up to 1 mM did not inhibit forskolin-stimulated adenylyl cyclase activity in cyc- cells (data not shown). Therefore, it would appear that cyc- cells do not express muscarinic receptors. Because Katada et al. (24) have shown that somatostatin via G activation inhibits adenylyl cyclase activity in cyc- cell membranes, further experiments were performed with somatostatin. As shown in Fig. 5, in the absence of ACV subdomains, somatostatin inhibited forskolin-stimulated adenylyl cyclase activity in a concentration-dependent manner. Addition of the C2 domain of ACV did not alter the inhibition of adenylyl cyclase by somatostatin (Fig. 5).

FIG. 3. The C1 (but not the C2) domain of ACV increases the ability of peptide MIII-4 to inhibit the forskolin-stimulated adenylyl cyclase activity of S49 cyc- membranes. A, S49 cyc- membranes (15 μg) were incubated in the absence or presence of peptide MIII-4 (0.1 mM) with and without the C1 domain (1.5 μM) of ACV for 30 min as described under “Experimental Procedures.” Thereafter, the membranes were assayed for adenylyl cyclase activity in the presence of forskolin (33 mM). Experiments were performed with membranes from both normal cells (−PTX) and cells exposed to pertussis toxin (+PTX, 10 μg/ml) for 24 h. B, S49 cyc- membranes were incubated under the same conditions as described for A, except that the C2 subdomain of ACV was used instead of C1. For A and B, all assays were performed in triplicates, and the data presented are the means ± S.E. of three experiments.

FIG. 4. The C1 (but not the C2) domain of ACV increases the ability of peptide MIII-4 to inhibit the Gα*-stimulated adenylyl cyclase activity of S49 cyc- membranes. A, S49 cyc- membranes (15 μg) were incubated in the absence or presence of peptide MIII-4 (0.1 mM) with or without the C1 domain as described under “Experimental Procedures.” These preincubated membranes were used in adenylyl cyclase with and without the C1 domain (1.5 mM carbachol at concentrations up to 1 mM) by themselves significantly altered AC activity that was stimulated by G or (Fig. 4A). However, the combination of MIII-4 and C1 inhibited AC activity by 60% (Fig. 4A). This effect was specific for the C1 domain of ACV since the C2 domain of ACV failed to alter AC activity in cyc- cells. That the inhibition observed in Fig. 4A was due to activation of endogenous Gi was demonstrated by the findings that when GTPγS was omitted from the preincubations of S49 cyc- membranes with or without MII-4 and/or the C1 domain, the inhibition shown in Fig. 4A was not observed (data not shown).

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Taken together, the data in Figs. 1–5 demonstrate that ACV and its C1 subdomain, which interacts with Goi (3, 4), enhance
C1 domain of ACV, which interacts with Goi, increases the guanine nucleotide exchange activity of a receptor mimetic peptide that activates Gi, whereas the C2 region of ACV, which interacts with Gao, increases the guanine nucleotide exchange activity of receptors that couple Gs (5). Therefore, it would appear that the interaction of the ACV domains with their respective Go subunits in the context of the heterotrimer is important for augmenting the signals transduced from receptors to the G proteins that they are coupled with.

Acknowledgments—We are deeply indebted to the following individuals who supplied several of the reagents: Dr. Alfred G. Gilman for the Ga cDNA, Dr. Randall Reed for the Gc cDNA, Dr. Wei-Jen Tang for the TP2000 strain of E. coli; Dr. Jeffrey I. Gordon for providing the plasmid pBB131 encoding S. cerevisiae N-myristoyltransferase, and Dr. Yoshihiro Ishikawa for the canine ACV cDNA.

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Fig. 5. The C1 domain of ACV, but not the C2 region, augments inhibition of adenylyl cyclase by low concentrations of somatostatin. 849 cyc cells (10 μg of protein) were assayed for adenylyl cyclase activity as described under “Experimental Procedures,” except that the activity was stimulated by 100 μM forskolin, and 10 μM GTP was present in the reaction mixture. Somatostatin at different concentrations was added to the assays performed in the absence and presence of 1.0 μM each C1 or C2 domain of ACV. Data are presented as percent of control of forskolin-stimulated activity and are the means ± S.E. of three experiments performed in triplicates.

the ability of the M4 muscarinic receptor mimetic peptide (MIII-4) and somatostatin receptors to activate Gi. These findings, along with our previous demonstration that the C2 domain of AC, which interacts with Goi, enhances the guanine nucleotide exchange factor activity of the β2-adrenergic receptor mimetic peptide (βIII-2) for Gs (5), indicate that the novel concept of AC modulating the ability of receptors to increase GTP-GDP exchange on G proteins is applicable to both G proteins that regulate its activity. In this context, it should be noted that Mukhopadhyay and Ross (25) have shown that phospholipase Cβ does not alter receptor-mediated GTP-GDP exchange on Gi. Because our experiments in cysc membranes with MIII-4 and activation of endogenous somatostatin receptors are consistent with the in vitro reconstitution data presented, it is unlikely that the differences between phospholipase Cβ (25) and AC (Ref. 5 and this study) are related to the use of lipid vesicles and/ or full-length receptor (25) versus the use of receptor mimetic peptides and reconstitution without lipid vesicles (Ref. 5 and this study). More likely, it is possible that different effectors modulate the activity of their respective G proteins in different manners. Additional studies in the future with various effectors of heterotrimeric G proteins should help resolve this possibility.

One of the most interesting aspects of our studies is that the

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*J. Biol. Chem.* 2000, 275:25915-25919.  
doi: 10.1074/jbc.M001687200 originally published online June 13, 2000

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