Differential Regulation of Type I and Type VIII Ca\(^{2+}\)-stimulated Adenylyl Cyclases by G\(_{i}\)-coupled Receptors \textit{in Vivo}\(^*\)

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Coupling of intracellular Ca\(^{2+}\) to cAMP increases may be important for some forms of synaptic plasticity. The type I adenylyl cyclase (I-AC) is a neural-specific, Ca\(^{2+}\)-stimulated enzyme that couples intracellular Ca\(^{2+}\) to cAMP increases. Since optimal cAMP levels may be crucial for some types of synaptic plasticity, mechanisms for inhibition of Ca\(^{2+}\)-stimulated adenylyl cyclases may also be important for neuroplasticity. Here we report that Ca\(^{2+}\) stimulation of I-AC is inhibited by activation of G\(_{i}\)-coupled somatostatin and dopamine D\(_{2}\) receptors. This inhibition is due primarily to G\(_{i}\), and not \(\beta y\) sub-units since coexpression of \(\beta y\)-binding proteins with I-AC did not affect somatostatin inhibition. However, \(\beta y\) released from G\(_{i}\) did inhibit I-AC, indicating that the enzyme can be inhibited by \(\beta y\) \textit{in vivo}. Interestingly, type VIII adenylyl cyclase (VIII-AC), another Ca\(^{2+}\)-stimulated adenylyl cyclase, was not inhibited by G\(_{i}\)-coupled receptors. These data indicate that I-AC and VIII-AC are differentially regulated by G\(_{i}\)-coupled receptors and provide distinct mechanisms for interactions between the Ca\(^{2+}\) and cAMP signal transduction systems. We propose that I-AC may be particularly important for synaptic plasticity that depends upon rapid and transient cAMP increases, whereas VIII-AC may contribute to transcriptional-dependent synaptic plasticity that is dependent upon prolonged, Ca\(^{2+}\)-stimulated cAMP increases.

Cross-talk between the Ca\(^{2+}\) and cyclic AMP signal transduction systems is one of several mechanisms important for synaptic plasticity (1–8). There are multiple mechanisms for Ca\(^{2+}\) regulation of adenylyl cyclases, including activation by protein kinase C (types I–III, V, and VII) (9–14), stimulation by calmodulin (CaM)\(^1\) and Ca\(^{2+}\) (types I and VIII) (2, 15–18), and inhibition by CaM-dependent protein kinase II (type III) (19, 20) and CaM-dependent protein kinase IV (type I) (67) as well as direct inhibition by Ca\(^{2+}\) (types V and VI) (21–24). The Ca\(^{2+}\)/CaM-stimulated adenylyl cyclases, type I (I-AC) and type VIII (VIII-AC) adenylyl cyclases (15, 25–27), are expressed in areas of the brain associated with learning and memory including the hippocampal formation (15, 28), and mice lacking I-AC have deficits in spatial memory and altered long-term potentiation (LTP) (7).

Mechanisms for attenuation of cAMP signals generated by the Ca\(^{2+}\)-stimulated adenylyl cyclases \textit{in vivo} have not been defined. Inhibition of the Ca\(^{2+}\)/CaM-stimulated adenylyl cyclases may be important during synaptic plasticity since optimal, not necessarily maximal, cAMP levels are necessary for certain forms of learning (4). Specifically, the sensitivity of Ca\(^{2+}\)-stimulated I-AC or VIII-AC to G\(_{i}\)-coupled receptor activation has not been reported. Although I-AC is inhibited by G\(_{i}\) \textit{in vitro} (29, 30), regulatory mechanisms determined with purified enzymes or membrane preparations are not necessarily operative \textit{in vivo}. For example, I-AC is stimulated by activated G\(_{i}\) in membranes; however, it is not stimulated by G\(_{i}\)-coupled receptor activation \textit{in vivo} (31). Additionally, G\(_{i}\) stimulation of III-AC is stimulated by Ca\(^{2+}\)/CaM in \textit{vitro} (32); however, \textit{in vivo}, elevations of intracellular Ca\(^{2+}\) inhibit G\(_{i}\) stimulation of III-AC (19, 20). Therefore, it was important to determine the responses of I-AC and VIII-AC to G\(_{i}\)-coupled receptors in intact cells. In this study, we report that I-AC, but not VIII-AC, is inhibited by activation of somatostatin or dopamine D\(_{2}\) receptors in HEK 293 cells. A comparison of I-AC with VIII-AC indicates that these two Ca\(^{2+}\)-stimulated enzymes have distinct regulatory properties.

EXPERIMENTAL PROCEDURES

Materials—3-Isobutyl-1-methylxanthine, carbachol, carbamylcholine chloride (carbachol), dopamine, forskolin, isoproterenol, serotonin, and somatostatin-14 were from Sigma. A23187 was purchased from Calbiochem. Pertussis toxin was obtained from List Biological Laboratories Inc. (Campbell, CA).

Cell Culture—Human embryonic kidney 293 (HEK 293) cells were grown at 37 °C in HEPES-buffered Dulbecco’s modified Eagle’s medium (H-DMEM) supplemented with 10% fetal bovine serum in a humidified 95% air, 5% CO\(_{2}\) incubator. HEK 293 cells stably expressing the long form of the dopamine D\(_{2}\) receptor (293-D\(_{2}\)) were generously provided by Dr. Kim Neve (Veterans Association Medical Center, Portland, OR). 293-D\(_{2}\) cells require maintenance in 2 μg/ml puromycin (Aldrich). Cell culture materials were from Life Technologies, Inc. unless otherwise indicated.

Expression of I-AC and VIII-AC in HEK 293 Cells—The I-AC cDNA clone was isolated from a bovine brain cDNA library as described (28). The cDNA clone for VIII-AC was generously provided by Dr. John Krupinski (Weis Center for Research, Geisinger Clinic, Danville, PA). Polyclonal populations of hygromycin (Calbiochem; 500 units/ml)-resistant 293 cells or 293-D\(_{2}\) cells were obtained by stable transfection of the pCEP4 expression vector (Invitrogen), pCEP4-IAC, or pCEP4-VIII-IAC by the calcium phosphate method (33). All stable cell lines were created from the same parental population of HEK 293 cells or 293-D\(_{2}\) cells. Expression of transfected adenylyl cyclases was determined by cAMP accumulation assays as described below.

cAMP Accumulation Assay—Changes in intracellular cAMP levels were measured by determining the ratio of \(^{3}H\)cAMP to a total ATP, ADP, and AMP pool in \(^{3}H\)adenine-loaded cells as described (34). This assay system allows for rapid and sensitive determination of relative changes in intracellular cAMP levels. While the ratios measured between assays may show some variation, the relative changes in cAMP levels between assays is quite reproducible. Briefly, as cells in six-well plates approached confluency (~90%), they were incubated in H-DMEM + 10% fetal bovine serum containing 2 μCi/ml \(^{3}H\)adenine (ICN) for 16–20 h. The next day, cells were aspirated, washed once with 150 mM NaCl, and incubated in buffer A (20 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 2.5 mM MgSO\(_{4}\), 2 mM CaCl\(_{2}\), 10 mM glucose, and...
2 mM sodium phosphate) or in H-DMEM + 1% penicillin/streptomycin containing the indicated effectors (e.g. A23187, carbachol, dopamine, forskolin, isoproterenol, serotonin, and somatostatin) plus 1 mM 3-isobutyl-1-methylxanthine. For extracellular Ca2+ dose responses, buffer A was made with varying concentrations of CaCl2. Reactions were terminated by aspiration and the addition of 1 ml of ice-cold 5% trichloroacetic acid, 1 mM cAMP. Culture dishes were maintained at 4°C for 1–4 h, and acid-soluble nucleotides were separated by sequential Dowex AG 50WX4 and neutral alumina chromatography as described (35). Reported data are the averages of triplicate determinations. Pertussis toxin (List Biological Laboratories Inc.), when used, was added to cells along with [3H]adenine for 16–20 h.

**Transient Coexpression of I-AC with the Carboxyl Terminus of β-Adrenergic Receptor Kinase 1 or Transducin-α in HEK 293 Cells**—The peptide mimetic construct encoding the carboxyl terminus of β-adrenergic receptor kinase 1 (βARK1-ct) in the pRK5 plasmid (36) was generously provided by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). A cDNA clone for the α subunit of human rod transducin was provided by Dr. Neil M. Nathanson (University of Washington, Seattle). The Gs-coupled serotonin receptor 5HT-7 cDNA (37) was a gift from Dr. Mark Hamblin (Veterans Association Medical Center, Seattle). Briefly, the night before transfection, cells were plated in 100-mm plates at 70% density. The following morning, each plate was transfected with 8 μg of total DNA (1 μg of 5HT-7; 2.5 μg of pCEP4, pCEP4-4AC, or pCEP4-IVAC; 4 μg of pCDNAIII (Invitrogen), pCDNAIII-transducin-α, or pRK-βARK1-ct; and 0.5 μg of RSV-β-galactosidase) in H-DMEM in the presence of 50–60 μl of Lipofectamine (Life Technologies Inc.). After 5 h, cells were rinsed with H-DMEM + 1% penicillin/streptomycin + 10% fetal bovine serum and maintained for 24 h under normal conditions. The following day, cells were split, pooled by transfection, and seeded into six-well culture dishes (one transfected plate/six-well dish) for cAMP assays as well as into 12-well plates (two wells/transfection) for β-galactosidase assays. The next morning, cells used for cAMP assays were labeled for 4–6 h with 2–3 μCi/well [3H]adenine. Just prior to the cAMP assay, companion cells for β-galactosidase assays were lysed in 500 μl of buffer B (100 mM KH2PO4, pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol) and frozen until use. cAMP and β-galactosidase assays were carried out as described below, and all raw data were normalized to the measured β-galactosidase signal for each transfection.

**β-Galactosidase Assay**—Lysates of transiently transfected cells (described above) were thawed and centrifuged at 16,000 × g for 10 min. 20 μl of the supernatant was combined with 100 μl of reaction buffer (100 mM NaH2PO4, pH 8.0, 1 mM MgCl2, 35 mM Galacton (Tropix Inc., Bedford, MA), and 100 mM β-galactoside) and incubated in the dark at room temperature for 60 min. During this incubation period, a 10% solution of Emerald (Tropix Inc.) in 0.2 N NaOH was prepared. These samples were frozen and thawed 5–6 times at 5-s intervals by a Berthold luminometer. Each well of lysed cells was assayed in duplicate, and the next day, the data were normalized to the measured β-galactosidase signal for each transfection.

**RESULTS**

**Inhibition of Ca2+-stimulated I-AC by Gs-coupled Receptors in Vivo**—To examine the effect of Gi-coupled receptors on I-AC in vivo, we carried out an initial screen of HEK 293 cells to determine if they express endogenous inhibitory receptors that couple to adenylyl cyclases. cAMP levels were elevated 6-fold by activation of endogenous β-adrenergic receptors with isoproterenol (31). Application of 1 μM somatostatin inhibited isoproterenol stimulation of the endogenous adenylyl cyclase(s) by ~50%, indicating the presence of somatostatin receptors (data not shown). Somatostatin receptors are seven-transmembrane domain proteins that typically couple to the Gi/Go class of heterotrimeric G proteins (39, 40).

**FIG. 1.** Gs-coupled receptors inhibit Ca2+/calmodulin-stimulated I-AC in HEK 293 cells. A, HEK 293 cells expressing I-AC (designated as I-AC here and in subsequent figures) were treated with the indicated concentrations of dopamine in the presence of 5 μM A23187 or vehicle. B, HEK 293 cells expressing the dopamine D2L receptor with I-AC (designated as D2L/I-AC here and in subsequent figures) were treated with the indicated concentrations of dopamine in the presence of 5 μM A23187 or vehicle. HEK 293 cells do not contain endogenous Ca2+-stimulated adenyly cyclase activity and are not represented. Relative cAMP accumulation was determined as described under Experimental Procedures. The data are the means ± S.D. of triplicate assays.
fected with I-AC. In these cells, dopamine maximally inhibited I-AC activity by 75%, with an IC50 of 50 nM (Fig. 1B).

The effect of somatostatin on Ca2+ -stimulated I-AC activity was examined at several concentrations of A23187 (Fig. 2A) and extracellular Ca2+ (Fig. 2B) to determine if somatostatin affected the Ca2+ sensitivity of I-AC. Ca2+ -stimulated I-AC activity was inhibited by ~50% at all concentrations of A23187 and Ca2+ examined. These data indicate that somatostatin inhibits I-AC without affecting its Ca2+ sensitivity.

Pertussis Toxin Abolishes Inhibition of I-AC by Gi-coupled Receptors—To determine whether inhibition of I-AC by somatostatin or dopamine is mediated via the Gi/Gs class of G proteins, HEK 293 cells expressing I-AC were treated with pertussis toxin. This toxin catalyzes the ADP-ribosylation of the α subunit of Gi/Gs (42–46) and un couples Gi/Gs from its receptors (47). Somatostatin inhibition of I-AC was completely abolished by pertussis toxin (Fig. 3A). Furthermore, dopaminergic inhibition of Ca2+ -st imulated I-AC was pertussis toxin-sensitive (Fig. 3B). Similar results were obtained when carbachol was used to stimulate I-AC (Fig. 4, A and B). These data indicate that somatostatin or dopamine inhibition of I-AC is most likely mediated by Gi or Gs.
were interested in assessing the role of the G protein \( G_i \) sub-units in hormonally driven inhibition since both \( G_i \) and \( G_{ia} \) inhibit I-AC in vitro (26, 30, 48, 49). To accomplish this, we carried out transient transfections of HEK 293 cells in which the COOH-terminal \( G_i \)-binding region of \( \beta \)-adrenergic receptor kinase 1 (\( \beta \)ARK1-ct) or the \( a \) subunit of human rod transducin (\( G_{ta} \)) was coexpressed with I-AC. IV-AC was used as a positive control for the expression of \( G_i \)-binding proteins. Since \( G_{ia} \) stimulation of IV-AC is potentiated by \( \beta y \) (50), effective expression of \( G_i \)-binding proteins should block this potentiation. Cellular expression of "peptide minigenes" encoding \( \beta y \)-binding pleckstrin homology domains of various proteins attenuates \( \beta y \) effects on phospholipase C, II-AC, and the mitogen-activated protein kinase pathway (36, 51, 52). Additionally, \( G_{ia} \) is an effective scavenger of free \( \beta y \) subunits in intact cells (53, 54).

In transient transfection experiments in which the \( G_{ia} \)-coupled 5HT-7 receptor was cotransfected, somatostatin potentiated serotonin stimulation of IV-AC, presumably through release of \( \beta y \) from \( G_{ia} \) (Fig. 5A). Coexpression of \( \beta \)ARK1-ct with IV-AC attenuated somatostatin-mediated potentiation of serotonin-stimulated IV-AC almost entirely (Fig. 5A). Under the
stimulation of I-AC in vitro can be inhibited by βγ (26, 30, 48, 49, 55) and since receptor activation of Gs releases βγ, is it possible that the insensitivity of I-AC to Gs-coupled receptor activation is due to βγ inhibition? To address this issue, we examined the sensitivity of I-AC to activation of the Gs-coupled 5HT-7 receptor in vivo when βγ-binding proteins were coexpressed. Coexpression of Gtbg with I-AC elicited a substantial (~4-fold) stimulation of I-AC by serotonin (Fig. 7B). Experiments in which βARK1-ct was used as the βγ scavenger gave similar results (Fig. 7D). In both cases, effective expression of βγ scavengers was determined with IV-AC as described (Figs. 7, A and C). These data suggest that βγ release from dissociating Gt heterotrimer inhibits I-AC.

**Ca2+ Stimulation of VIII-AC Is Not Inhibited by Gγ-coupled Receptors**—To determine whether Ca2+ stimulation of VIII-AC is also regulated by Gγ-activating hormones, VIII-AC-expressing cells were treated with 500 nm somatostatin in the presence of increasing concentrations of A23187. VIII-AC was stimulated ~7-fold by 10 μM A23187 in the presence of 1.8 mM extracellular Ca2+ (Fig. 8). Concentrations of somatostatin as high as 500 nm, which inhibited I-AC, did not inhibit VIII-AC at any concentration of A23187 (Fig. 8) or extracellular Ca2+ (data not shown) examined. Stimulation of VIII-AC in vivo by forskolin was also insensitive to somatostatin (data not shown).

To determine if VIII-AC is insensitive to other Gγ-coupled receptors, cells stably coexpressing the dopamine D2L receptor with VIII-AC were treated with increasing concentrations of dopamine in the presence or absence of A23187. Ca2+-stimulated VIII-AC was only inhibited 15% by 1 μM dopamine (Fig. 9). The insensitivity to somatostatin but slight inhibition by dopamine probably reflect differences in receptor density. The density of endogenous somatostatin receptors in HEK 293 cells is ~18 fmol/mg of protein (56), while the D2L cells used in this study express ~1500–2000 fmol/mg of protein. The D2L receptor density in the cell lines used in this study was ~10-fold higher than the D2 receptor density in the striatum, which expresses the highest levels of D2 receptors in the brain (57). Since the same stock of HEK 293 cells or dopamine D2L receptor-expressing cells was used for expression of I-AC or VIII-AC, variation in the number of somatostatin or D2L receptors present in I-AC- or VIII-AC-expressing cells cannot account for the difference in Gs sensitivity. Furthermore, somatostatin did not inhibit VIII-AC in four independent HEK 293 cell lines stably expressing VIII-AC. These data strongly suggest that VIII-AC is insensitive to Gγ-coupled receptor stimulation in vivo.

Gγ and Ca2+ Do Not Synergize to Stimulate VIII-AC—Since I-AC and VIII-AC respond quite differently to Gγ-coupled receptors in HEK 293 cells, it was of interest to compare the sensitivities of these two enzymes to Gγ-coupled receptors and Ca2+ in vivo. Both I-AC and VIII-AC were stimulated by Ca2+ alone, but neither was activated by isoproterenol, a β-adrenergic agonist (Fig. 10). I-AC was stimulated by isoproterenol when intracellular Ca2+ was elevated with A23187. In contrast, isoproterenol did not produce any additional stimulation of VIII-AC beyond that caused by A23187 alone (Fig. 10). Stimulation of I-AC by coapplication of Ca2+ and isoproterenol was strongly inhibited by somatostatin, whereas it had no effect on VIII-AC activities (data not shown).

Somatostatin Inhibits Ca2+-stimulated cAMP Accumulation in Primary Cortical and Hippocampal Neuron Cultures—Since I-AC is neuronal-specific (25) and expressed in the cortex and hippocampus (28), we were interested in determining whether or not somatostatin inhibits Ca2+-stimulated cAMP levels in cultured neurons. Primary cultures of cortical and hippocam-
Pal neurons were prepared from day 1 rat pups. Treatment of these cultures with 1 mM somatostatin produced a substantial inhibition of cAMP levels stimulated with 1 mM A23187, demonstrating that Ca$_{2+}$-stimulated cAMP synthesis in the hippocampus and cortex is sensitive to Gi-coupled hormones (Fig. 11).

**DISCUSSION**

Although Ca$_{2+}$ stimulation of specific adenyl cyclases in the brain may contribute to neuroplasticity (3, 5–8, 28), mechanisms for inhibition of adenyl cyclases may be equally important. In most cells, increases in cAMP in response to extracellular and intracellular signals are transient. Mechanisms for attenuation of cAMP signals include inhibition of adenyl cyclases by Gi-coupled receptors (18), CaM kinase inhibition of specific adenyl cyclases (19), and cAMP hydrolysis by phosphodiesterases (58). The objective of this study was to determine if I-AC or VIII-AC is inhibited by Gi$_1$-coupled receptors in vivo.

In this study, we discovered that I-AC is inhibited in vivo by activation of Gi$_1$-coupled receptors including somatostatin and dopamine D$_2$L receptors. Inhibition was apparently mediated through the G/G$_o$ class of heterotrimeric G proteins since it was blocked by pertussis toxin. In particular, we presume that inhibition was mediated by Gi, because Gi$_{1}$ and Gi$_{3}$ are expressed at relatively high levels in HEK 293 cells compared with G$_{o2}$ (56). Furthermore, G$_{o2}$ inhibition of I-AC in vitro is 10-fold less potent than that elicited by Gi$_1$ (30). Mechanisms for receptor-coupled inhibition of I-AC in vivo have not been defined, but could include contributions from activated G$_{i}$ and/or by since both have been shown to inhibit I-AC in vitro (26, 29, 30, 49, 55). Our data suggest that receptor-stimulated inhibition of I-AC is due primarily to activated G$_{i}$, since coexpression of βy-binding proteins did not prevent somatostatin inhibition. Parallel experiments with IV-AC coactivated with serotonin and somatostatin illustrated that βy was effectively inhibited by coexpression of the same βy-binding proteins. 

**FIG. 7.** Expression of βy-binding proteins with I-AC generates stimulation by activation of the Gi$_1$-coupled 5HT-7 receptor in vivo. HEK 293 cells were transiently transfected with both RSV-β-galactosidase and the 5HT-7 receptor, pCDNAIII, Gt$_{1}$, or βARK1-ct; as well as pCEP4, pCEP4-I-AC, or pCEP4-IV-AC as described under “Experimental Procedures.” Cells transfected with Gt$_{1}$ are denoted as Gt, and cells transfected with βARK1-ct are denoted as PH domain. A, IV-AC-transfected cells with or without Gt$_{1}$ coexpressed were treated with 10 μM serotonin (5-hydroxytryptamine (5-HT)) in the presence or absence of 500 nM somatostatin (SOM). B, I-AC-transfected cells with or without Gt$_{1}$ coexpressed were treated with or without 10 μM serotonin. C, IV-AC-transfected cells with or without βARK1-ct coexpressed were treated with 10 μM serotonin in the presence or absence of 500 nM somatostatin. D, I-AC-transfected cells with or without βARK1-ct coexpressed were treated with or without 10 μM serotonin. Data for IV-AC are expressed as percent cAMP accumulation in the absence of somatostatin, with this level being set as 100%. Data for I-AC are shown as percent of basal cAMP accumulation, with the base-line cAMP level set as 100%. The data are the means ± S.D. of triplicate determinations and were subtracted for endogenous cAMP levels and corrected for transfection efficiency using β-galactosidase.
Release of inhibitory bg from Gs may explain why I-AC is not activated by Gs-coupled receptors in vivo. Infact, sequestration of bg released from Gs caused stimulation of I-AC by Gs-coupled receptor activation in vivo. The differing effects of bg sequestration following Gs versus G, receptor activation could be because of distinct bg subunit compositions for Gs or Gi. Interestingly, using the yeast two-hybrid system, Yan and Gautam (59) have shown that there are substantial differences in the interactions between II-AC and bg 1–5. In particular, bg1 interacts with II-AC three to four times more strongly than bg4 and at least twice as strongly as bg3 and bg5. It may be that different bg isoforms determine, in large part, the potency with which a given bg complex affects adenylyl cyclases in vivo. Alternatively, bg release from Gi may be functionally inconsequential with regard to I-AC since the dominant mechanism for Gi-mediated inhibition is through the release of Gia.

Mammalian brain expresses at least two CaM-stimulated adenylyl cyclases, I-AC and VIII-AC, both of which are activated by intracellular Ca2+1. These enzymes are expressed in the hippocampus (15, 28), and I-AC knockout mice show residual Ca2+1-stimulated adenylyl cyclase activity consistent with the properties of VIII-AC (7, 60). Surprisingly, VIII-AC was not inhibited by activation of somatostatin receptors or high levels of exogenously expressed dopamine D2L receptors. In addition, VIII-AC was not stimulated by Gs-coupled receptors in vivo in the presence or absence of elevated intracellular Ca2+1. A com-
The physiologic function of these enzymes and the relationship between their regulatory properties and neuroplasticity may become apparent when mutant mice lacking VIII-AC become available.

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