Inhibition of G Protein-coupled Receptor Signaling by Expression of Cytoplasmic Domains of the Receptor*

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The third intracellular domain (3i) of G protein-coupled receptors plays a major role in the activation of G proteins. Alterations in this region of the receptor can affect receptor/G protein coupling efficiency and specificity. We recently reported (Luttrell, L. M., Cotecchia, S., Ostrowski, J., Kendall, H., Lefkowitz, R. J. (1993) Science 259, 1453–1457) that coexpression of the 3i domain of the α1b adrenergic receptor (AR) (α1b3i) specifically inhibited α1bAR-mediated inositol phosphate production, with no effect on D1a dopamine receptor (D1a-DR)-mediated cAMP production. Similarly, expression of the 3i domain of D1a-DR (D1a3i) inhibited D1a-DR-mediated cAMP production but did not affect α1bAR-mediated inositol phosphate accumulation. This suggests that peptides derived from a G protein-coupled receptor might serve as antagonists of receptor/G protein interactions. The present studies were performed to test the generality as well as the specificity of this phenomenon. The effect of expression of the second intracellular domain (2i), the 3i domain, and the fourth intracellular domain (4i) of α1bAR on second messenger generation mediated by the α1bAR, the M1 muscarinic cholinergic receptor (M1-AChR), and the D1a-DR was examined. Although the α1b2i domain had no effect on receptor/G protein coupling for any receptor tested, the α1b3i domain inhibited signaling mediated by α1bAR and M1-AChR but not by D1a-DR, while the α1b4i domain inhibited signaling mediated by each of the receptors. To investigate the generality of 3i domain-induced inhibition of receptor activity further, the 3i domains of two Gαi-coupled receptors (α1bAR and M1-AChR) and two Gαq-coupled receptors (α2AAR and 5HT2AAR) were tested for effects on the second messenger generation mediated by each of the receptors. In each case, the homologous 3i domain caused significant inhibition (40–60%), while the 3i domain of the receptor coupled to the same G protein also decreased receptor/G protein coupling. In contrast, receptor/G protein coupling appeared unaffected by expression of 3i domains derived from receptors coupled to different G proteins. The α1b3i domain-provoked inhibition of homologous receptor signaling was surmountable at high receptor density, and assays using a phorbol response element/reporter gene construct detected a weak enhancement of basal second messenger generation in cells expressing the α1b3i domain alone. These data demonstrate that disruption of receptor/G protein coupling by 3i domain peptides 1) can be generalized to both Gαi- and Gαq-coupled receptors and 2) likely results from the ability of these peptides to act as competitive weak partial agonists at the receptor/G protein interface.

The family of receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) contains members from several classes including adrenergic receptors, muscarinic cholinergic receptors, rhodopsin, the visual color opsins, several neurotransmitter receptors, and many peptide receptors. G protein-coupled receptors share a common putative structural topography composed of seven transmembrane domains separated by four extracellular regions and four intracellular regions (1). The extracellular regions form pockets in which specific agonists can interact or bind (2, 3). Mutagenesis studies have demonstrated that intracellular regions, particularly the 3i domain, interact with G proteins to stimulate effector systems (4–6). Studies utilizing chimeras of α1bAR and β2 adrenergic receptors (α1bAR, β2AR) have shown that the 3i domain provides structural determinants for specificity of receptor/G protein interaction (7). Similarly, substitution of a portion of the 3i domain of the α2AAR, a phospholipase C stimulatory Gαi-coupled receptor (8–12), into the corresponding region of the Gαq-coupled β2AR results in a chimera capable of coupling to Gi (13, 14).

Noting the critical role of the 3i domain in receptor/G protein coupling, we recently demonstrated (15) that cellular expression of the 3i domains of α1bAR or of the Gαq-coupled adenylyl cyclase stimulatory D1a-DR (16) results in specific inhibition of agonist-stimulated second messenger generation mediated via the homologous receptor. This suggests that disruption of receptor/G protein interaction may represent a novel form of receptor antagonism with possible therapeutic applications.

Although considerable evidence implicates the 3i domain of several G protein-coupled receptors as containing major determinants of selectivity in receptor/G protein coupling (13), other intracellular domains also apparently play a role. Peptides derived from the 2i, 3i, and/or 4i domains of the β2AR (17, 18), α1bAR (19, 20), M1-AChR (19), and rhodopsin (21) have been shown to interact with G proteins in vitro. The present study explores the generality, specificity, and possible mechanism of the inhibitory effects of intracellular receptor domains on receptor-mediated signal transduction. The effects of cellular expression of the 2i, 3i, and 4i domains of the α1bAR on homolo-

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gous receptor signal transduction are described. The effects of the S1 domains from two Gq-coupled (αqAR and M3AR) and two Gq-coupled (αqAR and M3AR) receptors on signaling mediated by each receptor are also assessed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The full-length cDNAs encoding the αqAR (22) and the human α3AR (1) were isolated in our laboratory. The cDNA encoding the D1-DR (16) was from M. G. Caron, and those encoding the human M1AR and M2AR (23) were from E. G. Peralta. The -356 fos/CAT construct (24) was provided by M. Z. Gilman. Rabbit polyclonal antisera against the α2AR domain, the α3AR domain, and the α3AR domain were provided by M. Breun.

Restriction endonucleases and T4 DNA ligase were from Promega; T7 sequencing kit, protein A-Sepharose, and deoxynucleotide triphosphates were from Pharmacia Biotech Inc. HEK-293 and COS-7 cells were from the American Type Culture Collection. Tissue culture media, fetal bovine serum, and gentamicin were from Life Technologies, Inc. 2-(4-[(Hydroxy-3-[3H]iodophenyl)ethylaminomethyl]tetralone ([125]IHEAT), 1benzyl 4-[3H]quinuclidinyl benzilate, [3H]butyrophilin, [3H]M1ACHR, [3H]Jadenine, [3H]cyclin 3, and [3H]CAMP ([3H]cytosine) were from Amersham. Unlabeled 125-iodinated peptides were raised to glutathione S-transferase fusion proteins containing peptide sequences corresponding to portions of the appropriate intracellular domains (31) and employed at 1:100 dilution in PBS for autoradiography and quantitated by phosphomager densitometry.

**Ligand Binding**—For saturation binding analysis, crude plasma membranes prepared from transfected HEK-293 or COS-7 cells as described (10) were resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and incubated with radioligand for 1 h at 25°C. Receptor expression was determined as binding of 1 nM [125]IHEAT to αqAR, 1 nM [3H]butyrophilin to α3AR, 1 nM [3H]Jadenine to M1AR, and 1 nM [3H]CAMP to M2AR. Nonspecific binding was less than 15% of each total binding.

**Inositol Phosphate and cAMP Production**—For measurement of total inositol phosphates (IPs), transfected HEK-293 or COS-7 cells were labeled for 18-24 h with myo-[3H]inositol (1-2 μCi/ml) in culture medium supplemented with 5% fetal bovine serum. After labeling, cells were washed with Dulbecco's phosphate-buffered saline, preincubated for 5-20 min in Dulbecco's phosphate-buffered saline supplemented with 20 mM LiCl and 1 mM CaCl2, and stimulated for 5 min with agonist as indicated. Following stimulation, total inositol phosphates were extracted as described (10) and separated on Dowex AG1-X8 columns (32). Total IPs were eluted with 1 mM ammonium formate, 0.1 mM formic acid and quantitated by liquid scintillation spectroscopy.

**Chloramphenicol Acetyltransferase Assay**—For determination of the effect of α3AR or α3AR domain peptide expression on phorbol response element activation, HEK-293 cells in 60-mm dishes (1 x 106 cells/dish) were transiently cotransfected with the indicated receptor or minigene construct plus pUC13 (-356 fos/CAT), a fusion plasmid consisting of the p106 CAT gene linked to the mouse c-fos gene promoter region beginning at -356 base pairs from the translation start site (24). Following transfection (48 h), monolayers were incubated for 2 h at 37°C in Dulbecco's phosphate-buffered saline supplemented with 20 mM LiCl and 1 mM CaCl2 with or without agonist, and CAT activity was determined. For measurement of cAMP production, cells were preincubated for 15 min at 37°C in Hanks balanced salt solution supplemented with 10 mM HEPES (pH 7.4) and 1 mM isobutyrylthiostatin and stimulated for 20 min with or without agonist. cAMP was extracted as described (33) and separated by sequential chromatography on Dowex AG50W-X4 and aluminum oxide columns using trace amounts of [14C]cAMP (10,000 dpm/column) to assess column recovery (34).

**RESULTS**

**Construction and Expression of DNA Plasmids**—A series of minigene constructs were prepared to allow transient expres-
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Fig. 1. Construction of G protein-coupled receptor-derived peptide minigenes. A, putative membrane topology of a G protein-coupled receptor. Shaded areas represent the approximate position of peptide sequences employed in the construction of intracellular domain minigenes. B, amino acid sequences of the α₂AR, α₃AR, M₁ACHR, and M₂ACHR intracellular domains employed in the construction of intracellular domain minigenes. The boxed residues represent receptor-derived sequences with numbered amino acid residues within each receptor and overall length of receptor-derived sequence as indicated. Each receptor-derived peptide sequence was preceded by methionine and glycine residues as described under "Experimental Procedures." Asterisks represent regions of sequence not shown. The DNA encoding each of the peptides was cloned into a minigene construct depicted schematically in the lower panel.

Effect of Expression of \( \alpha_1 \)AR Intracellular Domains on Homologous and Heterologous Receptor Signal Transduction—Transient transfection of cultured HEK-293 and COS-7 cells has been used to simultaneously coexpress multiple recombinant receptors, G protein subunits, and effector molecules or peptides, allowing the interaction between recombinant and endogenous components of signal transduction pathways to be studied (8–10, 35). The effects of coexpression of the \( \alpha_1 \)B₁i, -3i, and -4i domain peptides on \( \alpha_1 \)AR-mediated IP production were determined. As shown in Fig. 4A, the \( \alpha_1 \)B₁i and \( \alpha_1 \)B₄i domain peptides inhibited \( \alpha_1 \)AR activity by 40–50% when individually coexpressed with receptor. The \( \alpha_1 \)B₃i domain was inactive even when coexpressed with either or both of the active peptides. When the \( \alpha_1 \)B₃i and \( \alpha_1 \)B₄i domains were expressed simultaneously, the inhibitory effect was greater than that produced by either domain peptide alone (70%). As shown in Fig. 4B, the \( \alpha_1 \)B₃i and \( \alpha_1 \)B₄i domain peptides were approximately additive in cells co-transfected with submaximally active amounts of the minigene constructs. Inhibition of receptor-mediated signal transduction by maximally active amounts of pRK\( \alpha_1 \)B₃i and pRK\( \alpha_1 \)B₄i, while less than additive, was greater than that observed with either plasmid alone. These data suggest the \( \alpha_1 \)B₃i and \( \alpha_1 \)B₄i domain peptides do not compete for the same intracellular binding site and are consistent with the data in Fig. 3 that demonstrate a differential
pattern of receptor specificity for inhibition by each of the two domain peptides.

Generality and Specificity of 3i Domain-induced Inhibition of G Protein-coupled Receptors—Of the intracellular domains tested, the α1B3i domain produced the most specific inhibition of receptor-mediated signal transduction. To determine the generality and specificity of 3i domain peptide-mediated inhibition of signal transduction, we prepared a series of four 3i domain minigene constructs (two derived from Gs-coupled receptors (α2AR and M1ACHR) and two derived from Gi-coupled receptors (α3AR and M1ACHR), as shown in Fig. 1A). The effect of each of the 3i domain peptides on each of the four receptors was then assessed.

In transiently transfected HEK-293 cells, Gi-coupled receptors mediated Bordetella pertussis toxin (PT)-insensitive stimulation of phosphatidylinositol hydrolysis. In contrast, activation of the Gi-coupled receptors did not produce a detectable increase in IP production, although a PT-sensitive inhibition of adenyl cyclase was observed (data not shown). By comparison, transiently transfected COS-7 cells exhibited both PT-sensitive stimulation of IP production mediated through the Gi-coupled receptors (Fig. 5A) and PT-sensitive stimulation of IP production mediated through the Gi-coupled receptors (Fig. 5B). The magnitude of IP accumulation produced by stimulation of these receptors was similar to what has been previously reported in stably transfected cells lines (36, 37). Subsequent experiments utilized Gi-mediated (PT-sensitive) IP production in HEK-293 and Gi-mediated (PT-sensitive) IP production in COS-7 cells in order to provide a consistent assay system for study of the effect of 3i domain peptides on receptor-mediated signal transduction.

The generality of the 3i domain peptide-induced inhibition was assessed by determination of the effect of each 3i domain peptide on signal transduction mediated by the receptor from which the peptide was derived. Table II summarizes the effects of the α1αi3i, α2αi3i, M13i, and M23i domain peptides on IP hydrolysis mediated by each homologous receptor. Maximal agonist-stimulated IP production was significantly inhibited (35–50%) in each case. These data indicate that attenuation of receptor-mediated signal transduction by coexpression of homologous 3i domain peptides represents a general phenomenon, extending to at least two Gi-coupled receptors (α2AR and M1ACHR), two Gi-coupled receptors (α3AR and M1ACHR), and, as previously described, a Gi-coupled receptor (D4DR) (15).

The specificity of the inhibition was determined by assaying the effects of each 3i domain on IP production mediated via each of the four receptors in transiently cotransfected HEK-293 or COS-7 cells. Fig. 6 depicts the effects of maximally active levels of each 3i domain on the agonist dose-response relationship for IP production mediated by one receptor, the α1AR. As shown in Fig. 6, A and C, coexpression of α1αi3i and M13i peptides produced a decrease in the apparent efficacy (i.e., maximal stimulatory effect) of epinephrine. In contrast, as shown in Fig. 6, B and C, coexpression of α1αi3i and M23i peptides produced an increase in basal IP production.
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A. Basal 
Epinephrine, 10 μM

B. Basal 
Carbachol, 100 μM

C. Basal 
FD, 1 μM

FIG. 3. Specificity of α1bAR intracellular domain peptide-mediated inhibition. HEK-293 cells transiently co-transfected with 1 μg/dish of pRKα1bAR (A), pRKMAChR (B), or pRKD1aDR (C) plus pRK5 vector, pRKα32i, pRKα33i, or pRKα34i (10 μg/dish) were assayed for basal and maximal agonist-stimulated IP or cAMP production as described. Epinephrine (Epi, 10 μM), carbachol (Carb, 100 μM), and fenoldopam (FD, 10 μM) were used to stimulate α1bAR, M1AChR, and D1aDR, respectively. Data represent the mean ± S.E. values from triplicate determinations from four to six separate experiments. Receptor expression did not vary significantly between control cells and cells coexpressing the α3i domain. All data are presented in arbitrary units such that 1 unit equals the basal amount of signal produced in control (pRK5 vector) co-transfected cells. An asterisk indicates *p < 0.05 compared with control value.

![FIG. 4. Effects of α1bAR intracellular domain peptides on α1bAR-mediated IP production. A. Effects of maximally active amounts of α1bAR intracellular domain peptides singly and in combination. HEK-293 cells were transiently co-transfected with pRKα1bAR (1 μg/dish) plus pRK5 vector, pRKα2i, pRKα3i, or pRKα4i, alone or in combination as indicated (10 μg of each construct/dish). Maximal epinephrine-stimulated (10 μM) IP production was determined as described. Data represent the mean ± S.E. values from triplicate determinations from four separate experiments. B. Additivity of α3i and α4i domain peptide effects at less than maximally active levels. Cells were transiently co-transfected with pRKα1bAR (1 μg/dish) plus the indicated amounts of pRKα3i, pRKα4i, or pRKα3i + pRKα4i. Maximal epinephrine-stimulated IP production was determined as described. Data represent the mean ± S.E. values from triplicate determinations from four separate experiments. All data are presented as percent of the epinephrine-stimulated increase in IPs over basal levels normalized to control (pRK5 vector) co-transfected cells. Basal IP production did not vary significantly between experimental conditions.](image)

6, B, and D. α23i and M33i peptides produced no significant effect on agonist efficacy. In Fig. 7, the effect of each of the 3i domain peptides on IP production mediated by each of the four receptors is depicted. The α1bAR-mediated IP production was significantly inhibited by expression of the α3i or M3i domains with little effect produced by expression of the α4i or M4i domains (Fig. 7A). M1AChR-mediated IP production was sensitive to expression of the M3i domain peptide and, to a lesser extent, to the α3i domain peptide (Fig. 7C). IP production mediated by the G,-coupled α1bAR and M1AChR was most sensitive to coexpression of the homologous 3i domain peptide with little effect produced by expression of nonhomologous 3i domain peptides (Fig. 7, B and D).

These data are summarized in Table III. In general, the greatest inhibition of receptor-mediated signal transduction was caused by the homologous 3i domain peptide. In addition, the α23i and M3i domain peptides inhibited IP production provoked by both G,-coupled receptors (α1bAR and M1AChR). When 3i domain peptides derived from receptors coupled to one G protein were coexpressed with receptors coupled to a different G protein, little or no inhibitory effect was observed. Thus, it appears that there is considerable specificity in the inhibition of receptor-mediated IP production by 3i domain peptides. The inhibition provoked by α23i and M3i domain peptides extends to other receptors coupled to a common G protein, while α3i and M4i domain peptides appear to inhibit the homologous
receptor in a highly specific manner.

Assessment of the Mechanism of Inhibition of Receptor-mediated Signal Transduction by 3i Domain Peptides—Any of several mechanisms might account for the observed inhibition of receptor-mediated IP production by coexpression of 3i domain peptides. If the 3i domain peptide directly activated the G protein, enhanced basal second messenger production might promote desensitization of the signal transduction pathway, resulting in a diminished response to receptor activation. Indeed, we have observed that stable expression of a constitutively active mutant of the β2AR α1β3i AR(S288–294) produced an increase in basal IP production as previously described (13); however no detectable increase in basal IP production occurred in cells expressing either the wild type α1βAR or the α1β3i AR peptide (Ref. 15 and data not shown). To determine if the α1β3i AR peptide could provoke a more subtle increase in the basal rate of P1 hydrolysis, HEK-293 cells were cotransfected with pRK5 vector, pRKαβAR, the constitutively active mutant plasmid pRKαβAR(S288–294), or pRKαβ3i AR, and a CAT reporter gene construct linked to a phorbol response element, pUC13/-356 fos/CAT. As shown in Fig. 8, expression of the constitutively active mutant α1βAR(S288–294) resulted in a substantial enhancement of CAT protein expression compared to control. Overexpression of wild type α1βAR also caused a modest increase, which was enhanced when cells were exposed for 2 h to epinephrine. Expression of the α1β3i AR peptide resulted in an increase in CAT expression comparable with that observed with the wild type α1βAR in the absence of agonist. These data suggest that the α1β3i AR peptide interacts directly with the G protein in a manner analogous to the wild type α1βAR resulting in an in-

**TABLE II**

Parameters of ligand binding and IP production in HEK-293 and COS-7 cells coexpressing G protein-coupled receptors and their homologous 3i domain peptides

| Receptor    | G protein | Co-transfected plasmid | Ligand binding | IP production |
|-------------|-----------|------------------------|----------------|--------------|
|             |           |                        | Rmax* pmol/mg | Basal         | Agonist-stimulated | Inhibition of Rmax% |
| α1bAR       | Gq        | pRK5                   | 1.2 ± 0.26    | 1.00         | 3.89 ± 0.26     | 53.6               |
| α2aAR       | Gq        | pRKαβ3i AR             | 1.29 ± 0.03   | 1.12 ± 0.03  | 2.46 ± 0.43    | 53.6               |
| M1aChR      | Gq        | pRK5                   | 1.55 ± 0.28   | 1.00         | 1.86 ± 0.16    | 53.6               |
| M2aChR      | Gq        | pRKαβ3i AR             | 1.15 ± 0.23   | 0.95 ± 0.04  | 1.26 ± 0.09    | 61.6               |

*Mean ± S.E. of triplicate determinations in four to six separate experiments.

**FIG. 5.** PT-insensitive and -sensitive IP production in transfected COS-7 cells. COS-7 cell cultures were transiently transfected with 1.0 µg of pRKαβAR, pRKαβM2aChR, pRKαβM1aChR, or pRKαβAChR. Cells were pretreated 18–24 h with (solid bars) or without (open bars) 100 ng/ml B. pertussis toxin. Cells were stimulated for 45 min at 37 °C with epinephrine (100 µM, cells expressing α1βAR), carbachol (100 µM, cells expressing M1aChR or M2aChR), or UK-14304 (1.0 µM, cells expressing α2aAR). IP production was determined and is expressed as the mean ± S.E. values from triplicate determinations from three to five separate experiments. Data are presented as the agonist-stimulated increase in IPs over basal levels in arbitrary units such that 1 unit equals the basal amount of IPs measured in nonstimulated cells. Basal levels of IP production in cells transfected with plasmids coding for receptors were not significantly different compared with each other or with that in cells transfected with control vector.
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FIG. 6. Effects of homologous and heterologous 3i domain peptides on α1BAR-mediated IP production. HEK-293 cells were transiently co-transfected with pRKα1BAR (1 μg/dish) plus 10 μg/dish pRK vector, pRKα1B3i (A), pRKα2A3i (B), pRKα3i (C), or pRKα3i (D), and the agonist dose-response relationships for epinephrine-stimulated IP production were determined as described. Data are presented as the percent of maximal epinephrine-stimulated IP production in control (pRK5 vector) co-transfected cells and represent the mean ± S.E. values from triplicate determinations from four to eight separate experiments.

FIG. 7. Specificity of 3i domain peptide-induced inhibition of G protein-coupled receptor-mediated PI hydrolysis. HEK-293 cells (A and C) or COS-7 cells (B and D) were transfected with 1 μg/dish pRKα1BAR (A), pRKα2AAR (B), pRKα3i (C), or pRKα3i (D) plus the indicated amount of either pRKα1B3i (open circle), pRKα2A3i (solid circle), pRKα3i (open triangle), or pRKα3i (filled triangle). Cells were stimulated for 45 min with the appropriate agonist (100 μM epinephrine for α1BAR, 1.0 μM UK-14304 for α2AAR, or 1.0 mM carbachol for M1ACHR or M2ACHR). Data are presented as the percent of maximal agonist-stimulated increase in IP production over basal observed in control (pRK5 vector) co-transfected cells. (Basal IP production did not significantly differ between experimental conditions.) Data represent the mean ± S.E. values of triplicate determinations from four to eight separate experiments. Receptor expression did not vary significantly between control cells and cells coexpressing the 3i domain peptides.

crease in basal PI turnover similar to, but smaller than, that observed with the constitutively active mutant receptor. Since the magnitude of the α1B3i domain-induced effect is no greater than that produced by the α1AAR, it is unlikely to promote sufficient desensitization to account for the observed attenuation of agonist-stimulated IP production in co-transfected cells. Furthermore, IP production in response to NaF, which directly activates G proteins, was not affected by coexpression of the α1B3i domain peptide, demonstrating that the downstream components of the signaling pathway are intact (data not shown). These data suggest that the 3i domain peptides inhibit receptor-mediated signal transduction by binding to the G protein in a manner analogous to the receptor, thereby potentially disrupting receptor/G protein interaction.

If the α1B3i domain peptide-mediated inhibition results from competition between peptide and activated hormone-receptor complex for a common binding site on the Ga subunit, the inhibition should be surmountable under conditions where hormone-receptor complex is present in relative excess. As shown in Fig. 9A, the inhibitory effects of the α1B3i domain peptide...
The mean percent inhibition of the maximal agonist-stimulated increase in IP production for HEK-293 or COS-7 cells expressing maximally active amounts of each 3i domain construct were calculated and assigned values from 0 to ++++ as indicated. Data are derived from four to eight separate experiments performed with each combination of receptor and 3i domain. 0 indicates <10% inhibition; + indicates 10–20% inhibition; ++ indicates 20–30% inhibition; +++ indicates 30–40% inhibition; ++++ indicates >40% inhibition.

Table III

| Receptor       | G protein | Co-transfected receptor domain |
|----------------|-----------|--------------------------------|
| α1AR           | G        | α1AR 3i | M1,3i | α1AR 3i | M1,3i |
| M1,2-AR       | G        | ++      | ++++  | 0      | +     |
| α1AR          | G        | +       | ++    | +++    | 0     |
| M1,2-ChR      | +        | 0       | +     | ++++   | 0     |

Fig. 8. Phorbol response element-induced CAT expression in transfected HEK-293 cells. HEK-293 cells were transiently co-transfected with pUC18k-356 fos/CAT (0.5–1 μg/dish) plus pRK5 vector, pRKα3iAR (S288–294), pRKα3iAR, or pRKα3iAR (2 μg/dish) as indicated. Basal and epinephrine (Epi) stimulated expression of CAT activity was determined as described 48 h after transfection. Data are presented in arbitrary units such that 1 unit equals the basal level of CAT activity detected in control (pRK5 vector) transfected cells and represents the mean ± S.E. values from triplicate determinations from four separate experiments.

were determined in HEK-293 cells coexpressing varying levels of α1AR. In cells expressing less than 1 pmol/mg membrane protein of α1AR, a 60% inhibition of maximal α1AR-mediated IP production was observed. Increasing receptor density diminished the degree of inhibition, abolishing it at receptor levels greater than 5 pmol/mg of membrane protein. As shown in Fig. 9B, coexpression of increasing amounts of α1β-3i domain peptide resulted in a small (2–3-fold) increase in the EC50 for epinephrine-stimulated IP production in cells expressing a constant high level of α1AR. The failure of increasing agonist concentration to fully surmount the inhibition at higher levels of α1β-3i domain peptide expression probably reflects saturation of all available α1βAR at agonist concentrations in excess of 1 μM. Beyond that point, a further increase in agonist concentration does not result in an increase in hormone-receptor complex (the entity that would be expected to compete with the α1β-3i domain peptide for binding to Ga subunits). Collectively, these data suggest that the α1β-3i domain peptide acts as a weak agonist at the level of the G protein, producing a small increase in the rate of PI hydrolysis and reversibly antagonizing receptor-mediated G protein activation.

**DISCUSSION**

In this study, the inhibition of G protein-coupled receptor-mediated second messenger generation resulting from coexpression of peptides derived from the intracellular domains of the receptor in intact cells is described. We have previously demonstrated, using a similar approach (15), that coexpression of the 3i domains of α1AR and D1,DR specifically attenuates homologous receptor-mediated signaling. This report characterizes the generality and specificity of this phenomenon by examining the effects of the 2i, 3i, and 4i domain peptides of α1AR and the 3i domain peptides of α2AR, M1,ACHR, and M1,ACHR on homologous and heterologous receptor signaling. In each case, the 3i domain peptide significantly inhibited signaling mediated by the homologous receptor, with inhibition of heterologous receptor activity (if any) restricted to receptors utilizing a common G protein. This demonstrates a high degree of specificity in 3i domain peptide-induced inhibition of receptor-mediated signal transduction and suggests that the receptor-G protein interface may represent a therapeutic target for future drug development.

Previous studies of the effects of receptor-derived or related peptides on G protein function in reconstituted systems have demonstrated that specific peptides may behave as receptor-like agonists, antagonists of receptor-mediated G protein activation, or partial agonists at the level of the G protein. For example, peptides derived from the COOH-terminal region of the β2AR 3i domain (17, 18), the M1,ACHR 3i domain, and the α2AR 3i domain (19) mimic receptor by directly activating G proteins in vitro. Peptides derived from the COOH-terminal region of the α2AR also apparently inhibit receptor/G protein interaction in membrane preparations (20), as do peptides derived from the 2i, 3i, and 4i domains of rhodopsin (21). In addition, an oligopeptide substance P analog has been shown to competitively antagonize receptor-mediated activation of G, G, and G, in phospholipid vesicles by binding to Ga subunits (39). The results of the present study, which unlike others has used intact cells, suggest that the α1β-3i domain peptide acts as a functional ligand with the G protein (G) since α1β-3i expression modestly enhances phorbol response element-promoted expression of CAT activity. The finding that the α1β-3i domain peptide-provoked inhibition of receptor-mediated IP production is sustained at high levels of receptor expression supports the hypothesis that inhibition results from competition between the 3i domain peptide and the hormone-receptor complex for G protein binding. Therefore, it appears that 3i domain peptides act as weak partial agonists at the level of the G protein.

While the data indicate that the α1β-3i domain peptide does interact directly with the G protein, we cannot exclude the alternative hypothesis that interaction between the 3i domain peptide and the receptor itself disrupts receptor conforma- tion so as to impair receptor/G protein coupling and to contribute to the observed inhibition of IP production. If such a mechanism were valid, the expected pattern of inhibition by 3i domain peptides would be relatively receptor-specific since the affinity of a 3i domain peptide for the other intracellular domains of the homologous receptor would probably be greatest. In contrast, inhibition resulting from 3i domain peptide binding to the G protein might be more broadly G protein-specific. Occupancy of the receptor/G protein interface by a compatible 3i domain peptide would be expected to decrease signal transduction mediated by any receptor coupled with that G protein. The observed pattern of inhibition (Table III) did not completely distinguish these alternatives. While three of the four 3i domain peptides possessed demonstrable activity against the other re-

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Fig. 9. Surmountability of α3β3I domain-induced inhibition of αμAR-mediated IP production. A, activity of α3β3I domain peptide in HEK-293 cells expressing different levels of αμAR. HEK-293 cells were transiently co-transfected with pRKαβ3I and maximal epinephrine-stimulated IP production determined. The mean level of receptor expression for each curve is shown and varied by <10% within an experiment. Data are presented as percent of the maximal epinephrine-stimulated increase in IP production observed in control (pRK5 vector) co-transfected cells and represent mean values for triplicate determinations in one of three separate experiments that gave similar results. B, effect of α3β3I domain peptide on the dose-response relationship for epinephrine-stimulated IP production in co-transfected HEK-293 cells. HEK-293 cells were transiently co-transfected with pRKαβ3I (2.0 µg/dish) plus pRK vector or pRKαβ3I as indicated, and the agonist dose dependence for epinephrine-stimulated IP production was determined as described. The mean level of αμAR expression was 3.55 ± 0.09 pmol/mg for all curves. Data are presented in arbitrary units such that 1 unit equals the basal amount of IP production observed in control (pRK5 vector) co-transfected cells and represent mean values for triplicate determinations in one of three separate experiments that gave similar results. Basal IP production was not significantly different between experimental conditions. EC50 values for epinephrine, determined by least squares regression analysis were as follows: control, 10.4 nM; 1 µg of pRKαβ3I, 11.6 nM; 3 µg of pRKαβ3I, 13.1 nM; 10 µg of pRKαβ3I, 28.5 nM.

... which couples to the same G protein, the most striking inhibition in most cases occurred when a 3i domain peptide was coexpressed with its homologous receptor. While this may reflect a direct interaction between 3i domain peptide and its homologous receptor, it might also reflect intrinsic differences in the affinity of receptor/G protein interaction between different classes of G protein-coupled receptors. Alternatively, other intracellular domains of some receptors may contribute differentially to receptor/G protein interaction allowing some receptors to more easily displace a nonhomologous 3i domain peptide from the Gs subunit binding site.

In conclusion, this study demonstrates that inhibition of receptor-mediated signal transduction by 3i domain peptides occurs in both Gs- and Gq-dependent pathways and in both adrennergic and muscarinic cholinergic systems. The specificity with which the 3i domain peptides inhibit receptor-mediated signaling suggests the feasibility of developing drugs that might exert inhibition at the level of receptor/G protein interaction rather than at the level of ligand-receptor binding.

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