Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3637–3641, April 1994
Biochemistry

Functionally active targeting domain of the β-adrenergic receptor kinase: An inhibitor of $G_{\beta\gamma}$-mediated stimulation of type II adenylyl cyclase

J. INGLESE*, L. M. LUTTRELL*, J. A. IÑIGUEZ-LLUHI†, K. TOUHARA*, W. J. KOCH*, AND R. J. LEFKOWITZ*

*Departments of Medicine and Biochemistry, Box 3821, Howard Hughes Medical Institute at Duke University Medical Center, Durham, NC 27710; and †Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235

Contributed by R. J. Lefkowitz, January 7, 1994

ABSTRACT The β-adrenergic receptor kinase (βARK) phosphorylates its membrane-associated receptor substrates, such as the β-adrenergic receptor, triggering events leading to receptor desensitization. βARK activity is markedly stimulated by the isoprenylated βγ subunit complex of heterotrimeric guanine nucleotide-binding proteins ($G_{\beta\gamma}$), which translocates the kinase to the plasma membrane and thereby targets it to its receptor substrate. The amino-terminal two-thirds of βARK1 composes the receptor recognition and catalytic domains, while the carboxyl third contains the $G_{\beta\gamma}$ binding sequences, the targeting domain. We prepared this domain as a recombinant His_6 fusion protein from *Escherichia coli* and found that it had both independent secondary structure and functional activity. We demonstrated the inhibitory properties of this domain against $G_{\beta\gamma}$ activation of type II adenylyl cyclase both in a reconstituted system utilizing Sf9 insect cell membranes and in a permeabilized 293 human embryonic kidney cell system. $G_{i\alpha}$-mediated inhibition of adenylyl cyclase was not affected. These data suggest that this His_6 fusion protein derived from the carboxyl terminus of βARK1 provides a specific probe for defining $G_{\beta\gamma}$-mediated processes and for studying the structural features of a $G_{\beta\gamma}$-binding domain.

The βγ subunit complex of heterotrimeric signal-transducing G proteins ($G_{\beta\gamma}$) regulates many effectors within the cell, simultaneously participating in the propagation and termination of cellular signaling (1). $G_{\beta\gamma}$ has been shown to activate specific subtypes of phospholipase C (2) and the atrial K^+ channel (3, 4) and can either stimulate or inhibit various isoforms of adenylyl cyclase (5). In addition, two members of the G protein-coupled receptor kinase (GRK) family (6), β-adrenergic receptor kinases 1 and 2 (βARK1 and βARK2), are targeted by $G_{\beta\gamma}$ to their membrane-associated receptor substrates to facilitate the phosphorylation of activated receptors and initiate the process of receptor desensitization. Molecular cloning has revealed four β and five γ subunit genes (7) which result in defined $G_{\beta\gamma}$ combinations (8).

Many protein kinases have a modular architecture containing a catalytic domain accompanied by regulatory domains. Regulatory sequences, for example, may contain zinc-finger-like motifs (9), leucine zippers (10), Src homology SH2/SH3 domains (11), or Ca^{2+}/calmodulin-binding domains (12) which allow these kinases to interact with other cellular components to further activate signaling pathways. The regulatory element in specific GRKs (e.g., βARK1, βARK2 and DrosGPRK1) that interacts with $G_{\beta\gamma}$ is located in the carboxyl terminus of the kinase (13, 14) (see Fig. 1*A*). Members of the GRK family which do not interact with $G_{\beta\gamma}$ lack homologous $G_{\beta\gamma}$-binding domains (6). Interestingly, rhodopsin kinase (RK) is isoprenylated in a manner similar to the γ subunit of $G_{\beta\gamma}$ (15), allowing the kinase to be translocated to the rhodopsin-rich membranes of the retina in a light-dependent fashion (16).

In an effort to develop a functionally active $G_{\beta\gamma}$-binding domain, we expressed the carboxyl-terminal domain of βARK1 in *Escherichia coli* as a His_6-tagged fusion protein. Here we describe the properties of this stable 27-kDa domain which retains the ability to form a complex with $G_{\beta\gamma}$ and inhibits the $G_{\beta\gamma}$ stimulation of type II adenylyl cyclase both in a reconstituted membrane system and in intact cells.

MATERIALS AND METHODS

Materials. The full-length cDNA encoding the human dopamine 1A receptor ($D_{1A}R$) was provided by Marc Caron (Howard Hughes Medical Institute, Duke University) and that for the rat type II adenylyl cyclase (17) by Randall Reed (Johns Hopkins University). Human embryonic kidney 293 (HEK-293) cells (ATCC CRL-1573) were from the American Type Culture Collection. Eagle's minimum essential medium, fetal bovine serum, and gentamicin were from GIBCO. Reduced streptolysin O was from Wellcome. Radioligands, including [^{3}H]cAMP, [^{3}H]SCH23390, [^{3}H]yohimbine, and [α-^{32}P]ATP were from New England Nuclear. Phentolamine was from CIBA-Geigy, *cis*-flupentixol from Research Biochemicals (Natick, MA), UK14304 from Pfizer Diagnostics, and fenoldopam (SKF82526) from SmithKline Beecham.

His_6 Fusion Proteins. His_6-RK carboxyl terminus (His_6-RKct) and His_6-βARK1 carboxyl terminus (His_6-βARK1ct), containing the terminal 91 amino acids of RK and the terminal 222 amino acids of βARK1, were prepared as follows. PCR primers corresponding to bases encoding amino acids 467–473 (Pro-Pro-Phe-Val-Pro-Asp-Ser) of RK and 468–474 (Pro-Pro-Leu-Ile-Pro-Pro-Arg) of βARK1 (5′ primers containing a *Bam*HI restriction site) and the inverse complement of bases encoding Pro-Ser-Ser-Lys-Ser-Gly-Met(stop) in RK and Arg-Gly-Ser-Ala-Asn-Gly-Leu(stop) in βARK1 (3′ primers containing a *Hin*dIII site) were used to amplify the carboxyl terminus of the respective kinases. The amplified fragment was ligated in frame into the *Bam*HI and *Hin*dIII sites of the pQE30 vector (Qiagen) and the resultant constructs were used to transform *E. coli* strain NM522. The fusion proteins were induced with isopropyl β-D-thiogalactopyranoside and purified on a nickel-chelate adsorbent resin (ProBond resin; Invitrogen) (18). Fusion proteins were eluted from the ProBond resin with 500 mM imidazole/50 mM sodium phosphate/300 mM NaCl/1 mM 2-mercaptoethanol, pH 6.0, and dialyzed against phosphate-buffered saline (20 mM sodium phosphate, pH 7.4/150 mM NaCl) containing 1 mM 2-mer-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: α_{2A}AR, α_{2A}-adrenergic receptor; βARK, β-adrenergic receptor kinase; $D_{1A}R$, dopamine 1A receptor; GRK, G protein-coupled receptor kinase; GTP[γS], guanosine 5′-[γ-thio]triphosphate; HEK, human embryonic kidney; r (prefix), recombinant; RK, rhodopsin kinase.

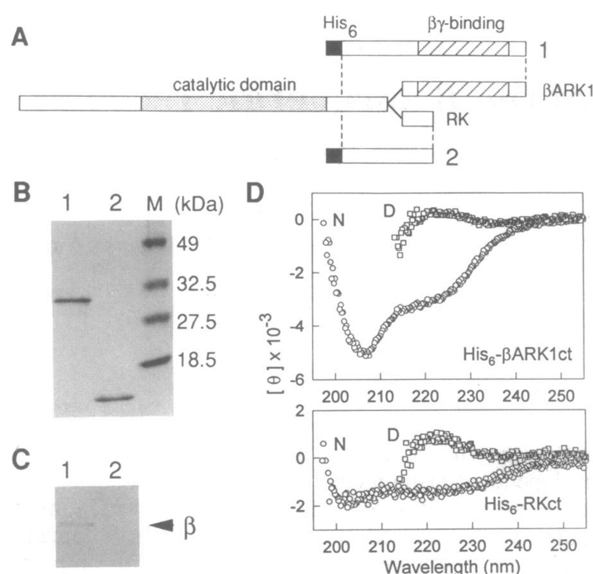

FIG. 1. Properties of the isolated carboxyl-terminal targeting domain of βARK1. (*A*) Domain structure of βARK1 and RK indicating the regions from which His_6-βARK1ct (construct 1) and His_6-RKct (construct 2) are derived. (*B*) SDS/PAGE of purified His_6-βARK1ct (lane 1) and His_6-RKct (lane 2) fusion proteins. Molecular size standards (lane M) are to the right. (*C*) $G_{\beta\gamma}$ binding to His_6-βARK1ct (lane 1) or His_6-RKct (lane 2) on nickel-agarose. $G_{\beta\gamma}$ bound to the resin was eluted, subjected to SDS/PAGE, transferred to nitrocellulose, and detected with an antibody to G_β. (*D*) CD spectra of His_6-βARK1ct and His_6-RKct (1.4 μM) in 20 mM phosphate, pH 6.0/100 mM NaCl without (N, native) or with (D, denatured) 6 M guanidine. The mean residue ellipticity, [θ] has units of degree·cm^2·$dmol^{-1}$.

captoethanol and 1 mM EDTA at 4°C. Further purification of the fusion proteins was achieved by anion-exchange chromatography [Mono Q FPLC (Pharmacia), 10–500 mM NaCl linear gradient]. Protein concentrations were determined by Bradford assay (Bio-Rad) with bovine serum albumin as a standard.

Detection of $G_{\beta\gamma}$ Binding to Fusion Proteins. The binding of $G_{\beta\gamma}$ to fusion proteins and the detection of bound $G_{\beta\gamma}$ were accomplished essentially as described (13, 14). In brief, purified bovine brain $G_{\beta\gamma}$ (183 nM) with or without the fusion protein (1 μM) in 60 μl of phosphate-buffered saline were incubated for 20 min on ice. Then, 20 μl of a 50% slurry of ProBond resin (Invitrogen) in phosphate-buffered saline was added and incubation was continued on ice for 15 min with gentle mixing. The beads were then washed three times with 500-μl aliquots of phosphate-buffered saline containing 0.01% Lubrol. The protein separation and the detection of $G_{\beta\gamma}$ were carried out as described (13, 14) except that 1% gelatin in Tris-buffered saline (10 mM Tris, pH 8.0/150 mM NaCl) was utilized for blocking.

Circular Dichroism (CD) Spectra of Fusion Proteins. The CD spectra of purified His_6-βARK1ct and His_6-RKct (1.4 μM each) were measured in 20 mM sodium phosphate, pH 6.0/100 mM NaCl from 260 to 200 nm at 0.25-nm resolution on an Aviv model 62DS CD spectrophotometer using a 1.0-cm-path-length cell. The spectra of denatured fusion proteins were also determined in 6 M guanidine hydrochloride. The solvent background was subtracted from each spectrum.

Reversal of the $G_{\beta\gamma}$ Potentiation of Recombinant $G_{s\alpha}$ ($rG_{s\alpha}$)-Stimulated Type II Adenylyl Cyclase Activity by His_6-βARK1ct. Assays for type II adenylyl cyclase were carried out essentially as described (19) with some modifications. Various amounts of the fusion proteins were preincubated in a volume of 15 μl in the presence or absence of Sf9-cell derived $rG_{\beta1\gamma2}$ for 5 min at 30°C and transferred to 4°C. The mixtures were then combined with 5-μl aliquots of Sf9 cell membranes containing type II adenylyl cyclase (20) that had been preincubated for 10 min at 30°C with guanosine 5′-[γ-thio]triphosphate (GTP)-[γS]-activated $rG_{s\alpha}$. The reactions were initiated by addition of 30 μl of an assay mixture containing the remaining cyclase assay components (5), which yielded final concentrations as follows: membrane proteins, 0.1 mg/ml; GTP-[γS]-activated $rG_{s\alpha}$, 30 nM; $rG_{\beta1\gamma2}$, 0.3 nM; $MgCl_2$, 10 mM.

Cell Culture and Transfection. The cDNAs encoding the human α_{2A} adrenergic receptor (α_{2A}AR) (21) and human D_{1A}R (22) were subcloned into pRK5 (23) for transient expression in HEK-293 cells (24). Transfections were carried out by coprecipitation of DNA with calcium phosphate (24), using the indicated amounts of DNA. Assays were performed 48 hr after the cultures were split. Where indicated, cells were treated with pertussis toxin (200 ng/ml) in growth medium for 18 hr prior to assay. To quantitate α_{2A}AR expression, saturation binding of 1 nM [^{3}H]yohimbine to plasma membranes was assayed as described (25), with 100 μM phentolamine to determine nonspecific binding. D_{1A}R expression was determined by saturation binding of 1 nM [^{3}H]SCH23390 with 1 μM *cis*-flupentixol to determine nonspecific binding.

Permeabilization of Cells with Streptolysin O and Assay of Adenylyl Cyclase Activity. Adherent monolayers of transfected HEK-293 cells in 24-well tissue culture plates were permeabilized by a modification of the procedure of Cuhna-Melo *et al.* (26). In brief, monolayers were washed once with 0.4 ml of KG buffer (139 mM potassium glutamate/5 mM glucose/20 mM potassium salt of Pipes/2.5 mM $MgCl_2$/1 mM EDTA, pH 7.4) and permeabilized by incubation for 5 min at 37°C in 0.2 ml of KG buffer containing reduced streptolysin O (0.25 unit equivalents/ml). This procedure resulted in the permeabilization of >95% of the cells as assessed by trypan blue staining. After permeabilization, monolayers were preincubated for 10 min at 37°C in 0.18 ml of peptide buffer (20 mM Hepes/2.5 mM $MgCl_2$/1 mM EDTA, 0.01% bovine serum albumin/0.1 mM 3-isobutyl-1-methylxanthine, pH 7.4) containing the indicated concentrations of fusion protein. The assay was initiated by the addition of 0.02 ml of concentrated adenylyl cyclase assay mix, containing adrenergic or dopaminergic agonists as indicated, directly to the peptide buffer to yield final concentrations as follows: phospho*enol*pyruvate, 2.7 mM; GTP, 53 μM; cAMP, 0.1 mM; ATP, 0.12 mM; pyruvate kinase, 4.0 units/ml; myokinase, 20 units/ml; [α-^{32}P]ATP, 20 μCi/ml (1 μCi = 37 kBq). Incubations were continued for 20 min at 37°C and terminated by the addition of 0.8 ml of stop solution (0.25% perchloric acid/0.1 mM cAMP with 20 nCi of [^{3}H]cAMP to serve as a tracer for the assessment of product recovery). [α-^{32}P]cAMP was determined as described (27). Accumulation of product under these assay conditions was linear for at least 30 min (data not shown).

RESULTS AND DISCUSSION

Properties of the Isolated βARK1 Carboxyl-Terminal Domain. Mammalian GRKs have related primary structures, with the catalytic domain flanked by an amino-terminal domain of conserved length and a carboxyl-terminal region of variable length (Fig. 1*A*). A GST fusion protein with the last 222 amino acids of βARK1 was previously used to demonstrate the presence of a $G_{\beta\gamma}$-binding domain in this region of the kinase (13). This fusion protein functioned as an inhibitor of $G_{\beta\gamma}$-stimulated βARK1 phosphorylation of rhodopsin (14) and as an inhibitor of odorant-induced, βARK2-mediated desensitization in permeabilized rat olfactory cilia (28).

A new fusion protein derived from the carboxyl-terminal $G_{\beta\gamma}$-binding domain of βARK1 was prepared in which the

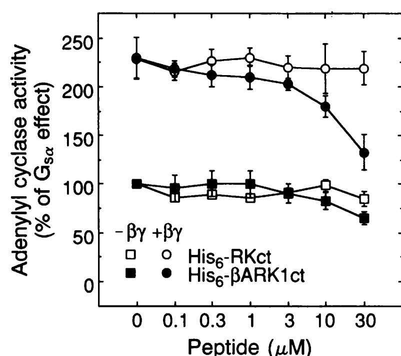

FIG. 2. Dose-dependent reversal of $rG_{\beta1\gamma2}$ potentiation of $rG_{s\alpha}$-stimulated type II adenylyl cyclase activity by His$_6$-βARK1ct. Sf9 membranes containing type II adenylyl cyclase were first incubated with GTP[γS]-bound $rG_{s\alpha}$ (final concentration, 30 nM) and then assayed for adenylyl cyclase activity in the presence (circles) or absence (squares) of $rG_{\beta1\gamma2}$ (final concentration, 0.3 nM) and the indicated concentrations of His$_6$-RKct (open symbols) or His$_6$-βARK1ct (filled symbols). Assays were carried out for 10 min at 30°C. Data represent mean ± SEM values for duplicate determinations of three separate experiments. The average type II adenylyl cyclase activity in the presence of $rG_{s\alpha}$ alone was 9.2 nmol·min^{-1}·mg^{-1}.

26-kDa GST moiety of the previously constructed GST-βARK1ct was replaced by 10 amino acids containing a hexahistidine (His$_6$) sequence (18). The resultant protein was expressed in *E. coli* and was rapidly purified by employing a nickel-chelate column and Mono Q FPLC. As a control, the carboxyl terminus of RK was also prepared as a His$_6$-tagged protein (Fig. 1*B*). His$_6$-βARK1ct but not His$_6$-RKct was shown to interact with $G_{\beta\gamma}$ in a binding assay where His$_6$-βARK1ct immobilized on a nickel-agarose column selectively bound bovine brain $G_{\beta\gamma}$ (Fig. 1*C*).

CD spectra of His$_6$-βARK1ct suggest that the fusion protein contains secondary structural features that can be denatured by 6 M guanidine (Fig. 1*D*) and that may reflect elements of a $G_{\beta\gamma}$-binding domain. His$_6$-RKct, in contrast, has a significantly different structure under similar conditions. The denatured His$_6$-βARK1ct was successfully refolded to give essentially the same spectrum as before denaturation (data not shown). This further supports the possibility that this region of βARK1 exists as an independent domain.

Effect of His$_6$-βARK1ct on $rG_{s\alpha}$-Stimulated type II Adenylyl Cyclase Activity in Sf9 Membranes. Characterized adenylyl cyclases differ in their regulation by free $G_{\beta\gamma}$ (5). Different types may be conditionally stimulated, inhibited, or insensitive to $G_{\beta\gamma}$. To ascertain whether His$_6$-βARK1ct could function as a general inhibitor of $G_{\beta\gamma}$-dependent processes other than translocation of the parent βARK1, this material was tested for its ability to inhibit $G_{\beta\gamma}$-dependent activation of type II adenylyl cyclase by a defined $G_{\beta\gamma}$, $G_{\beta1\gamma2}$. Type II adenylyl cyclase expressed in Sf9 membranes and preincubated with 30 nM GTP[γS]-activated $rG_{s\alpha}$ was treated with 0.3 nM $rG_{\beta1\gamma2}$ plus either His$_6$-βARK1ct or His$_6$-RKct at various concentrations. His$_6$-βARK1ct specifically inhibited the effect of $rG_{\beta1\gamma2}$ on $rG_{s\alpha}$-stimulated cyclase activity with an $IC_{50} \approx 15$ μM, whereas the control material, His$_6$-RKct, had no effect over the range of concentrations tested (Fig. 2).

Effect of His$_6$-βARK1ct on Receptor-Mediated Activation of Type II Adenylyl Cyclase in Permeabilized HEK-293 Cells. HEK-293 cells have been used to simultaneously coexpress multiple recombinant receptors, G-protein subunits, effector molecules, or peptides, allowing the interactions between recombinant and endogenous components of signal transduction pathways to be studied (24, 29). HEK-293 cells do not contain type II adenylyl cyclase (30) and show negligible conditional stimulation of endogenous adenylyl cyclases by exogenous $G_{\beta\gamma}$ (31). To determine whether His$_6$-βARK1ct would antagonize the conditional stimulation of type II adenylyl cyclase by $G_{\beta\gamma}$ released from endogenous G proteins, we developed an assay for adenylyl cyclase activity in streptolysin O-permeabilized HEK-293 cells simultaneously cotransfected with the cDNAs encoding G_i-coupled α_{2A}AR (21), the G_s-coupled D_{1A}R (22), and type II adenylyl cyclase (17). In HEK-293 cells coexpressing G_i-coupled receptors and the type II cyclase, conditional stimulation of the cyclase can be observed only in the presence of active $G_{s\alpha}$ (29). α_{2A}AR, unlike many other G_i-coupled receptors, exhibits a small degree of productive interaction with G_s (29, 32) and is capable of activating type II adenylyl cyclase without another source of active $G_{s\alpha}$, probably by releasing $G_{\beta\gamma}$ from G_i while simultaneously interacting with G_s to provide active $G_{\alpha s}$ subunits (29).

HEK-293 cells expressing α_{2A}AR and D_{1A}R exhibited a pertussis toxin-insensitive activation of the endogenous adenylyl cyclase in response to the dopamine agonist fenoldopam (Fig. 3*A*). The α_2-adrenergic agonist UK14304 did not activate the endogenous cyclase. Simultaneous coexpression

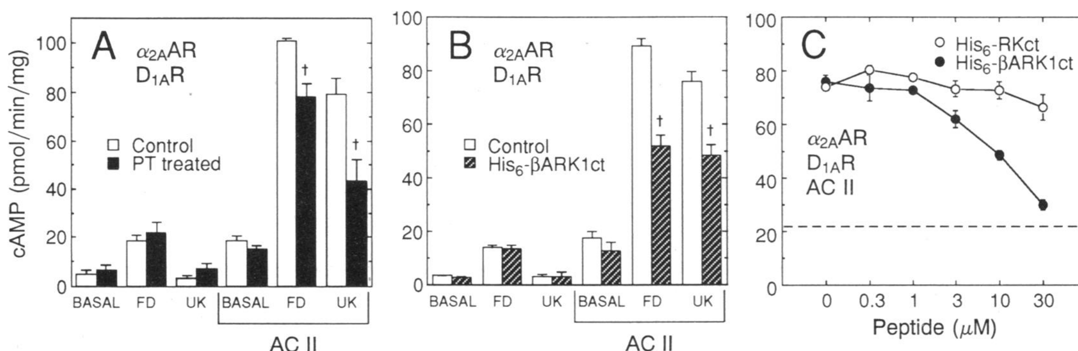

FIG. 3. Effect of His$_6$-βARK1ct and His$_6$-RKct on the activation of endogenous and transiently overexpressed type II adenylyl cyclase (AC II) in permeabilized HEK-293 cells. HEK-293 cells were transiently cotransfected with pRK5-α_{2A}AR (0.25 μg per 100-mm dish), pRK5-D_{1A}R (0.75 μg per 100-mm dish), and pcDNA-1-AC II (10.0 μg per 100-mm dish) as indicated in each panel. Levels of α_{2A}AR and D_{1A}R expression were approximately 0.80 and 2.80 pmol/mg of membrane protein, respectively. Final concentrations of fenoldopam (FD) and UK14304 (UK) were 1 μM and 10 μM, respectively. (*A*) Effect of pertussis toxin (PT) on activation of endogenously expressed adenylyl cyclase and transiently coexpressed AC II. (*B*) Effect of His$_6$-βARK1ct (10 μM) on activation of endogenous and coexpressed AC II. Data shown in *A* and *B* represent the mean ± SEM values for three to five separate experiments, each performed in triplicate. †, Less than control, $P < 0.01$. (*C*) Concentration dependence of His$_6$-βARK1ct (●) and His$_6$-RKct (○) effects on the α_{2A}AR-mediated activation of coexpressed AC II. The broken line indicates the level of adenylyl cyclase activity measured in control wells in the absence of added agonist or peptide. Data shown represent mean ± SEM values for triplicate determinations in one experiment which was replicated three additional times with similar results.

of α_{2A}AR, D_{1A}R, and type II adenylyl cyclase resulted in a marked enhancement of fenoldopam-stimulated adenylyl cyclase and the appearance of UK14304-stimulated adenylyl cyclase activity. Both effects were partially inhibited by preincubation with pertussis toxin. The pertussis toxin sensitivity of fenoldopam-stimulated adenylyl cyclase activity in cells coexpressing α_{2A}AR, D_{1A}R, and type II adenylyl cyclase is consistent with a contribution of free $G_{\beta\gamma}$ derived from G_i due to some constitutive activity of the markedly overexpressed α_{2A}AR, even in the absence of agonist (33). Cells coexpressing only D_{1A}R and type II cyclase exhibited a less marked enhancement of fenoldopam-stimulated cyclase activity which was insensitive to pertussis toxin (data not shown).

The ability of His$_6$-RKct and His$_6$-βARK1ct to inhibit receptor-mediated activation of either endogenous adenylyl cyclase or coexpressed type II adenylyl cyclase was determined in HEK-293 cells expressing both α_{2A}AR and D_{1A}R. Neither fusion protein produced a significant effect on D_{1A}R-mediated stimulation of the endogenous cyclase. In contrast, preincubation with 10 μM His$_6$-βARK1ct resulted in a significant inhibition of both the D_{1A}R-mediated and the α_{2A}AR-mediated conditional activation of coexpressed type II cyclase which resembled the effects of pertussis toxin (Fig. 3*B*). The dependence of inhibition of α_{2A}AR-mediated stimulation of type II adenylyl cyclase on the concentration of His$_6$-βARK1ct and the control, His$_6$-RKct, was assessed (Fig. 3*C*). At the highest concentration assayed, 30 μM, His$_6$-βARK1ct inhibited 70.8 ± 7.7% ($n = 3$) of α_{2A}AR-stimulated type II cyclase activity, compared with 16.3 ± 2.8% ($n = 3$) inhibition by His$_6$-RKct. The approximate IC_{50} for His$_6$-βARK1ct was 10.8 ± 2.3 μM ($n = 5$), which is in good agreement with the data from the membrane reconstitution experiment (Fig. 2). These data suggest that the conditional stimulation of type II adenylyl cyclase by $G_{\beta\gamma}$ released from endogenous G_i is specifically antagonized by His$_6$-βARK1ct with an IC_{50} similar to the one obtained with exogenously supplied $G_{\beta\gamma}$ subunits.

Effects of Pertussis Toxin and His$_6$-βARK1ct on Receptor-Mediated Inhibition of the Endogenous Adenylyl Cyclase in Permeabilized HEK-293 Cells. In contrast to the stimulatory role of $G_{\beta\gamma}$ subunits released from G_i in the conditional activation of type II adenylyl cyclase, G_i-coupled-receptor-dependent *inhibition* of adenylyl cyclase appears to be mediated, at least in some cases, by $G_{i\alpha}$ (34). Treatment with pertussis toxin, which catalyzes the ADP-ribosylation of G_i and prevents its functional interaction with receptors and therefore its dissociation, would not be expected to differentiate between the effects of G_i-coupled receptors mediated by $G_{i\alpha}$ and those mediated by $G_{\beta\gamma}$. Thus, treatment with pertussis toxin would be expected to inhibit both α_{2A}AR-mediated conditional stimulation of type II adenylyl cyclase and inhibition of the endogenously expressed adenylyl cyclase in HEK-293 cells. His$_6$-βARK1ct, however, which apparently inhibits conditional activation of the type II cyclase through its ability to bind $G_{\beta\gamma}$, would not be expected to impair effects of G_i-coupled receptors mediated by $G_{i\alpha}$. HEK-293 cells coexpressing α_{2A}AR and D_{1A}R exhibited a significant inhibition of fenoldopam-stimulated adenylyl cyclase activity in the presence of the α_2AR agonist UK14304; the inhibition was completely reversed by pertussis toxin (Fig. 4*A*). The His$_6$-βARK1ct had no effect on α_{2A}AR-mediated inhibition of the endogenous cyclase (Fig. 4*B*). These data suggest that His$_6$-βARK1ct can discriminate between α_{2A}AR effects mediated by the $G_{\beta\gamma}$ subunits of G_i (e.g., the conditional stimulation of type II adenylyl cyclase) and those mediated by $G_{i\alpha}$ (e.g., inhibition of D_{1A}R-stimulated endogenous adenylyl cyclase).

In summary, His$_6$-βARK1ct is an independent $G_{\beta\gamma}$-binding regulatory domain of βARK1 which, when expressed separately from holo-βARK1, retains a putative subdomain structure (Fig. 1*C*) and $G_{\beta\gamma}$-binding ability (Fig. 1*B*). Moreover, His$_6$-βARK1ct may be a general inhibitor of $G_{\beta\gamma}$-mediated processes, as it can block $G_{\beta\gamma}$-dependent activation of type II adenylyl cyclase in Sf9 membranes. His$_6$-βARK1ct introduced into permeabilized whole cells blocks receptor-mediated activation of cotransfected type II adenylyl cyclase, confirming that in cells, the activation of the type II cyclase is dependent upon release of $G_{\beta\gamma}$ from endogenous G proteins. Furthermore, His$_6$-βARK1ct discriminates between $G_{i\alpha}$-mediated (pertussis toxin-sensitive inhibition of endogenous adenylyl cyclase) and $G_{i\beta\gamma}$-mediated (pertussis toxin-sensitive activation of type II adenylyl cyclase). These findings, together with our recent demonstration of whole-cell inhibition of $G_{i\beta\gamma}$-mediated phospholipase C activity in COS-7 cells following the cellular expression of the βARK1 carboxyl terminus (35), suggest that these reagents may provide a research tool to define the potential $G_{\beta\gamma}$ dependence of a wide variety of cell regulatory processes. In addition, the CD spectra reported here suggest that His$_6$-βARK1ct may be amenable to more detailed structural analysis (such as NMR spectrometry and x-ray crystallography) which would provide insights into $G_{\beta\gamma}$-binding-domain structure and facilitate the search for functional $G_{\beta\gamma}$-binding domains in other proteins.

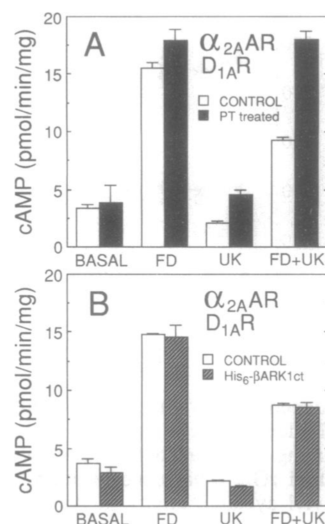

FIG. 4. Effect of pertussis toxin (PT) and His$_6$-βARK1ct on α_{2A}AR-mediated inhibition of endogenous adenylyl cyclase in permeabilized HEK-293 cells. (*A*) Reversal of α_{2A}AR-mediated inhibition of D_{1A}R-induced stimulation of endogenous adenylyl cyclase activity by PT treatment. (*B*) Effect of His$_6$-βARK1ct (10 μM) on α_{2A}AR-mediated inhibition of D_{1A}R-induced stimulation of endogenous adenylyl cyclase activity. AC assay mixture contained either no agonist (basal), 1 μM fenoldopam (FD), 10 μM UK14304 (UK), or both agonists (FD+UK). Data shown represent the mean ± SEM values for three separate experiments, each performed in triplicate.

Note Added in Proof. A domain referred to as the pleckstrin homology domain (36, 37) has been identified within the βARK1 carboxyl terminus as well as in various other proteins (38, 39). GST fusion proteins containing a region of these pleckstrin homology domains are capable of binding $G_{\beta\gamma}$ *in vitro* (40), suggesting that pleckstrin homology domains may interact with $G_{\beta\gamma}$ *in vivo*, thereby bringing previously unappreciated proteins under G-protein control.

We thank Dr. R. T. Premont and Dr. T. G. Oas for helpful discussions. This study was supported in part by National Institutes of Health Grant HL16037 (R.J.L.). J.A.I.-L. is a recipient of a Howard Hughes Medical Institute predoctoral training award.

1. Clapham, D. E. & Neer, E. J. (1993) *Nature (London)* **365**, 403–406.

2. Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jakobs, K. H. & Gierschik, P. (1992) *Eur. J. Biochem.* **206**, 821–831.
3. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. (1987) *Nature (London)* **325**, 321–326.
4. Wickman, K. D., Iñiguez-Lluhi, J. A., Davenport, P. A., Taussig, R., Linder, M. E., Krapivinsky, G. B., Gilman, A. G. & Clapham, D. E. (1994) *Nature (London)*, in press.
5. Tang, W.-J. & Gilman, A. G. (1992) *Cell* **70**, 869–872.
6. Inglese, J., Freedman, N. J., Koch, W. J. & Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 23735–23738.
7. Iñiguez-Lluhi, J. A., Kleuss, C. & Gilman, A. G. (1993) *Trends Cell Biol.* **3**, 230–236.
8. Pronin, A. N. & Gautam, N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6220–6224.
9. Bell, R. M. & Burns, D. J. (1991) *J. Biol. Chem.* **266**, 4661–4664.
10. Dorow, D. S., Devereux, L., Dietzsch, E. & De Kretser, T. (1993) *Eur. J. Biochem.* **213**, 701–710.
11. Birge, R. B. & Hanafusa, H. (1993) *Science* **262**, 1522–1524.
12. Schulman, H. & Hanson, P. I. (1993) *Neurochem. Res.* **18**, 65–77.
13. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G. & Lefkowitz, R. J. (1992) *Science* **257**, 1264–1267.
14. Koch, W. J., Inglese, J., Stone, W. C. & Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 8256–8260.
15. Inglese, J., Glickman, J. F., Lorenz, W., Caron, M. G. & Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 1422–1425.
16. Inglese, J., Koch, W. J., Caron, M. G. & Lefkowitz, R. J. (1992) *Nature (London)* **359**, 147–150.
17. Feinstein, P. G., Schrader, K. A., Bakalyar, H. A., Tang, W.-J., Krupinski, J., Gilman, A. G. & Reed, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10173–10177.
18. Janknecht, R., Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A. & Stunnenberg, H. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8972–8976.
19. Iñiguez-Lluhi, J. A., Simon, M. I., Robishaw, J. D. & Gilman, A. G. (1992) *J. Biol. Chem.* **267**, 23409–23417.
20. Taussig, R., Quarmby, L. M. & Gilman, A. G. (1993) *J. Biol. Chem.* **268**, 9–12.
21. Lorenz, W., Lomasney, J. W., Collins, S., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1990) *Mol. Pharmacol.* **38**, 599–603.
22. Dearry, A., Gingrich, J. A., Falardeau, P., Fremeau, R. T., Bates, M. D. & Caron, M. G. (1990) *Nature (London)* **347**, 72–75.
23. Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Lawn, R. M., Vehar, G. A. & Gorman, C. (1986) *Biochemistry* **25**, 8343–8347.
24. Luttrell, L. M., Ostrowski, J., Cotecchia, S., Kendall, H. & Lefkowitz, R. J. (1993) *Science* **259**, 1453–1457.
25. Cotecchia, S., Ostrowski, J., Kjelsberg, M. J., Caron, M. G. & Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 1633–1639.
26. Cuhna-Melo, J. R., Gonzaga, H. M. S., Ali, H., Huang, F., Huang, K.-P. & Beaven, M. A. (1989) *J. Immunol.* **89**, 2617–2625.
27. Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548.
28. Boekhoff, I., Inglese, J., Schleicher, S., Koch, W. J., Lefkowitz, R. J. & Breer, H. (1994) *J. Biol. Chem.* **269**, 37–40.
29. Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R. & Bourne, H. R. (1992) *Nature (London)* **356**, 159–161.
30. Premont, R. T. (1994) *Methods Enzymol.*, in press.
31. Premont, R. T., Chen, J., Ma, H.-W., Ponnapalli, M. & Iyengar, R. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9809–9813.
32. Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R. & Liggett, S. B. (1992) *J. Biol. Chem.* **267**, 15795–15801.
33. Ren, Q., Kurose, H., Lefkowitz, R. J. & Cotecchia, S. (1993) *J. Biol. Chem.* **268**, 16483–16487.
34. Taussig, R., Iñiguez-Lluhi, J. & Gilman, A. G. (1993) *Science* **261**, 218–221.
35. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M. & Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269**, 6193–6197.
36. Mayer, B. J., Ren, R., Clark, K. L. & Baltimore, D. (1993) *Cell* **73**, 629–630.
37. Haslam, R. J., Koide, H. B. & Hemmings, B. A. (1993) *Nature (London)* **363**, 309–310.
38. Shaw, G. (1993) *Biochem. Biophys. Res. Commun.* **195**, 1145–1151.
39. Musacchio, A., Gibson, T., Rice, P., Thompson, J. & Saraste, M. (1993) *Trends Biol. Sci.* **18**, 343–348.
40. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G. & Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269**, in press.