Phosphorylation of Chick Heart Muscarinic Cholinergic Receptors by the β-Adrenergic Receptor Kinase†

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ABSTRACT: Previous studies have demonstrated that muscarinic cholinergic receptors (mAChR) become markedly phosphorylated when intact cardiac cells are stimulated with a muscarinic agonist. This process appears to be related to the process of receptor desensitization. However, the mechanism of agonist-induced phosphorylation of mAChR is not known. In situ phosphorylation studies suggested that agonist-induced phosphorylation of mAChR may involve the participation of a receptor-specific kinase and/or require agonist occupancy. These observations regarding phosphorylation and desensitization of mAChR are similar to observations made for β-adrenergic receptors. Recent studies have indicated that homologous desensitization of β-adrenergic receptors may be due to the phosphorylation of these receptors by a novel protein kinase that only recognizes the agonist-occupied form of the receptors. As muscarinic receptors are structurally homologous to β-adrenergic receptors, we have initiated studies to identify the protein kinase responsible for the phosphorylation of muscarinic receptors by determining whether the chick heart muscarinic receptor would serve as a substrate for the β-adrenergic receptor kinase (β-AR kinase). We report that the purified and reconstituted chick heart muscarinic receptor serves as an excellent substrate in vitro for the β-AR kinase. Phosphorylation of mAChR receptors by the β-AR kinase was only observed in the presence of a muscarinic receptor agonist and was prevented in the presence of antagonist. Both the extent of phosphorylation (3–4 mol of P/mol of receptor) and the phosphoamino acid composition of the mAChR after incubation in vitro with β-AR kinase were similar to the characteristics of agonist-induced phosphorylation of mAChR in situ. As many of the properties of the in vitro phosphorylation of mAChR with β-AR kinase and the agonist-induced phosphorylation of mAChR in situ are similar, the results suggest that β-AR kinase or a similar enzyme may phosphorylate the mAChR in intact cells. The present results also extend our knowledge of the properties of β-AR kinase by directly demonstrating that this novel enzyme effectively phosphorylates a nonadrenergic receptor. The results support the hypothesis that this protein kinase may be of general importance in the regulation of receptors coupled to the modulation of adenylyl cyclase.

Recent studies have implicated protein phosphorylation as a widespread mechanism in the regulation of receptor function (Sibley et al., 1987; Huganir & Greengard, 1987). In the class of neurotransmitter receptors, it has been demonstrated that β_1- (Stadel et al., 1983), β_2- (Strasser et al., 1986a), and α_1- (Leeb-Lundberg et al., 1985) adrenergic as well as nicotinic (Miles et al., 1987) and muscarinic (Kwatra & Hosey, 1986) cholinergic receptors undergo phosphorylation in intact cells. In each case, phosphorylation has been implicated in the process of receptor desensitization; however, the exact mechanisms involved are not known in every instance.

Cardiac muscarinic cholinergic receptors (mAChR) have been shown to undergo a striking increase in phosphorylation in situ in response to stimulation of chick cardiac preparations with muscarinic agonists (Kwatra & Hosey, 1986; Kwatra et al., 1987). This process appears to result in a decrease in the affinity of the receptors for agonists, consistent with the concept that phosphorylation of these receptors is associated with desensitization (Kwatra et al., 1987). However, the mechanism of phosphorylation is not known. Attempts to identify the protein kinase(s) responsible for this phosphorylation suggested that the kinase was not one of the well-characterized protein kinases that are regulated by second messengers such as cyclic nucleotides, Ca^{2+}, or diacylglycerols (Kwatra et al., 1987). The results of these in situ phosphorylation studies suggested that the phosphorylation of the chick heart muscarinic receptors *either* required agonist occupancy of the receptors *or* was mediated by a receptor-specific protein kinase.

The phosphorylation and desensitization of cardiac muscarinic receptors by agonist-induced events have many parallels to the regulation of adrenergic receptors by phosphorylation (Stadel et al., 1983; Strasser et al., 1986a; Benovic et al., 1986, 1987a). Recent studies concerning the mechanism of desensitization of adrenergic receptors have implicated the participation of a novel protein kinase that only recognizes as substrate the agonist-occupied forms of certain adrenergic receptors (Benovic et al., 1986). This enzyme, referred to as the β-adrenergic receptor kinase (β-AR kinase), does not appear to be regulated by any known second messengers and has been shown to effectively phosphorylate the agonist-occupied forms of the β_2- (Benovic et al., 1986, 1987a) and the α_2-adrenergic receptors (Benovic et al., 1987a). The enzyme does not phosphorylate the agonist-unoccupied forms of these

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receptors, or does so only poorly. Several lines of evidence support the involvement of β-AR kinase in homologous desensitization of the β_2-adrenergic receptor. In vitro studies suggest that receptor phosphorylation by β-AR kinase uncouples receptor interaction with the stimulatory guanine nucleotide binding protein by a mechanism that may also involve an arrestin-like protein (Benovic et al., 1987c). Removal of the proposed β-AR kinase phosphorylation sites on the β_2-adrenergic receptor by site-directed mutagenesis dramatically slows the ability of isoproterenol to induce homologous desensitization in stably transfected Chinese hamster fibroblast cells (Bouvier et al., 1988). Finally, recent studies have demonstrated that heparin, a potent β-AR kinase inhibitor, prevents rapid homologous desensitization of β_2-adrenergic receptors in permeabilized A431 cells (Lohse et al., 1989). Other evidence suggests that this kinase may only recognize those adrenergic receptors that are coupled to the modulation of adenylyl cyclase, since it phosphorylates the β_2- and α_2-adrenergic receptors, which stimulate and attenuate adenylyl cyclase, respectively, but does not phosphorylate the α_1-adrenergic receptor, which is coupled to stimulation of phosphoinositide hydrolysis (Benovic et al., 1987a). On the other hand, indirect evidence has suggested that the enzyme β-AR kinase may be of general importance in regulating the phosphorylation of receptors coupled to adenylyl cyclase, as other cyclase-coupled receptors, such as the prostaglandin E_1 (Strasser et al., 1986b) and somatostatin (Mayor et al., 1987) receptors, induce translocation of β-AR kinase from the cytosol to the plasma membrane when activated. However, a direct demonstration of the phosphorylation of nonadrenergic receptors by the kinase has not yet been possible.

Cardiac mAChR, like β- and α_2-adrenergic receptors, couple to regulation of adenylyl cyclase (Murad et al., 1962). In addition, recent molecular cloning studies have indicated that muscarinic receptors have structural homologies to adrenergic receptors (Kubo et al., 1986; Peralta et al., 1987a,b; Bonner et al., 1987). In view of these similarities, and the similarities in the properties of agonist-induced phosphorylation and desensitization of muscarinic and adrenergic receptors, it seemed possible that the agonist-mediated phosphorylation of muscarinic receptors might occur via mechanisms similar to those implicated for adrenergic receptors. To test this possibility, we have determined whether purified chick cardiac muscariic receptors could serve as substrates for β-AR kinase in vitro.

Experimental Procedures

Purification of Receptors and the β-Adrenergic Receptor Kinase. β-AR kinase was purified from bovine cerebral cortex with minor modification of a previously described procedure (Benovic et al., 1987b). The specific activity of the purified enzyme was ~50–80 nmol of P_i min^{-1} (mg of protein)$^{-1}$ (Benovic et al., 1987b). The purified kinase had no detectable cAMP-dependent or Ca^{2+} phospholipid-dependent protein kinase present as determined by using histones as substrate. Muscarinic receptors were purified from chick ventricles with the muscarinic receptor affinity column (Haga & Haga, 1983) as previously described (Kwatra & Hosey, 1986). The specific activities of the purified receptors, measured with the muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB), were ~1 nmol of [^{3}H]QNB bound/mg of protein (Kwatra & Hosey, 1986).

Reconstitution of Chick Heart mAChR. In order to prepare receptor for phosphorylation studies, the mAChR was reconstituted out of detergent solution into phospholipid vesicles, since phosphorylation reactions mediated by β-AR kinase are inhibited by the detergent digitonin (Benovic et al., 1987b) used in the receptor purification procedure. Furthermore, as β-AR kinase is very sensitive to inhibition by ionic strength (Benovic et al., 1987b), it was necessary to utilize buffers low in NaCl. The method of reconstitution was modified from that described for porcine brain mAChR (Florio & Sternweis, 1985; Haga et al., 1986). Total lipids from chick heart, prepared according to the method of Folch et al. (1957), were used for reconstitution. An aliquot of chick heart lipids (1.2 mg) was dried under nitrogen and was suspended in 200 μL of 0.18% deoxycholate and 0.04% cholate in buffer A (20 mM Hepes, pH 8.0, 1 mM EDTA, 160 mM NaCl) (Haga et al., 1986). This suspension was sonicated for 10 min at 4 °C at maximum power in a cup horn sonicator (W-385 sonicator, Heat Systems Ultrasonics, Inc., Farmingdale, NY). For reconstitution, 10–15 pmol of receptor in 10 mM Tris-HCl, pH 7.5, and 0.05% digitonin was first mixed with oxotremorine (to yield a final concentration of 10 mM) in buffer A and allowed to stand at room temperature for 20 min. As previously observed (Florio & Sternweis, 1985; Haga et al., 1986), the oxotremorine was necessary to stabilize the receptor during the reconstitution process. Subsequently, 80 μL of sonicated lipids was added, and the final volume was adjusted to 0.2 mL with buffer A. The mixture was vortexed and kept on ice for 10–15 min. Reconstitution was achieved by passing the mixture over a small (1-mL) Extracti-gel column that had been pretreated with 0.2% bovine serum albumin in buffer A and washed with buffer B (20 mM Hepes, pH 8.0, 1 mM EDTA, 40 mM NaCl). The Extracti-gel column was eluted with buffer B, and 200-μL fractions were collected. This process also resulted in the removal of free oxotremorine by the Extracti-gel resin. Reconstituted receptors were assayed for [^{3}H]QNB binding by a filtration assay containing (500 μL) 20 mM potassium phosphate, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 10–15 μL of reconstituted receptor, and 10 nM [^{3}H]QNB, ± 10 μM atropine. The assay mixture was incubated at 37 °C for 75 min and filtered on GF/F glass filter papers (Florio & Sternweis, 1985).

Phosphorylation of mAChR by β-AR Kinase. For in vitro phosphorylation of the purified mAChR by the β-adrenergic receptor kinase, the reaction mixtures (50 μL) consisted of 10–15 μL of reconstituted receptor (0.3–1.5 pmol) in buffer B, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$, ± 1 mM carbachol, ± 0.1 mM atropine, and 5 μL of β-AR kinase (~50 ng). The reactions were started by adding 0.1 mM [γ-^{32}P]ATP (1–4 cpm/fmol) and were incubated at 30 °C for the times indicated. The reactions were stopped by adding 25 μL of SDS sample buffer and were then electrophoresed on SDS gels containing 8.5% polyacrylamide. After the gels were fixed, stained, and dried, the phosphorylation of the receptors was visualized by autoradiography. The receptor bands were excised from the dried gels and counted, and the stoichiometries of phosphorylation were calculated from the amount of receptor loaded onto each gel lane and the specific activity of the ATP. Phosphoamino acid analysis was performed as previously described (Kwatra et al., 1987).

Materials. The materials used for the purification of chick heart mAChR were obtained from the sources described previously (Kwatra & Hosey, 1986). Oxotremorine was purchased from K & K Labs, Inc., Plainview, NY. McN-A343 was from Research Biochemicals, Inc., Wayland, MA. Carbachol and deoxycholic and cholic acids were from Sigma Chemical Co., St. Louis, MO. [^{3}H]QNB was from Amersham, Arlington Heights, IL.

Results and Discussion

To test the possibility that the chick heart mAChR could

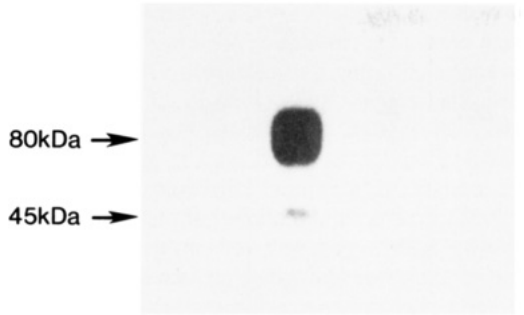

FIGURE 1: Phosphorylation of the purified and reconstituted chick heart muscarinic receptor by the β-adrenergic receptor kinase. Phosphorylation reactions were performed for 45 min as described under Experimental Procedures. Shown is an autoradiogram obtained after electrophoresis of the phosphorylated proteins on an SDS–polyacrylamide gel. All reactions contained the β-adrenergic receptor kinase and 0.35 pmol of mAChR (80-kDa peptide). Other additions were as noted.

serve as a substrate for β-AR kinase, purified receptors were reconsittuted out of detergent solution and into phospholipid vesicles and subjected to phosphorylation conditions in the presence and absence of muscarinic receptor ligands. As shown in Figure 1 (lane 1), incubation of the mAChR with β-AR kinase *in the absence of agonist* did not result in mAChR phosphorylation. However, addition of the agonist carbachol resulted in a marked phosphorylation of the receptor (80-kDa protein) (lane 2). Similar results were obtained in the presence of the agonist oxotremorine (data not shown). [In addition to the main receptor band of 80 kDa, a minor peptide of ~45 kDa was also phosphorylated in an agonist-dependent fashion; a similar phosphopeptide was also observed in in situ studies of agonist-induced phosphorylation of mAChR (Kwatra et al., 1987). This phosphopeptide may be a breakdown product of the mAChR.] That the phosphorylation of mAChR by β-AR kinase was dependent on the presence of an agonist ligand, and not merely receptor occupancy, was determined by the demonstration that no phosphorylation occurred when the mAChR was incubated with β-AR kinase and the muscarinic receptor antagonist atropine (Figure 1, lane 3). Furthermore, atropine prevented the agonist-dependent phosphorylation of mAChR when the receptor was incubated with β-AR kinase in the simultaneous presence of atropine and carbachol (Figure 1, lane 4). In addition, no significant receptor phosphorylation was observed in reactions containing the weak partial agonist McN-A343 (data not shown), which also does not induce mAChR phosphorylation in intact cells (Kwatra and Hosey, unpublished observations). Moreover, no phosphorylation of the mAChR was observed when the receptor was incubated in the presence or absence of cholinergic ligands under phosphorylating conditions but in the absence of β-AR kinase (not shown). Thus, no endogenous receptor kinase copurifies with the mAChR. Although the purified β-AR kinase does not appear to be contaminated by other protein kinases such as cAMP-dependent protein kinase or protein kinase C, to obtain further evidence that β-AR kinase was the enzyme responsible for the agonist-dependent phosphorylation of the mAChR, we demonstrated that heparin (1 μM) completely inhibited the carbachol-dependent phosphorylation catalyzed by β-AR kinase (R. M. Richardson, data not shown). Heparin has been recently demonstrated to be a potent inhibitor of β-AR kinase (K_i = 0.15 μM; Benovic et al., 1989). While

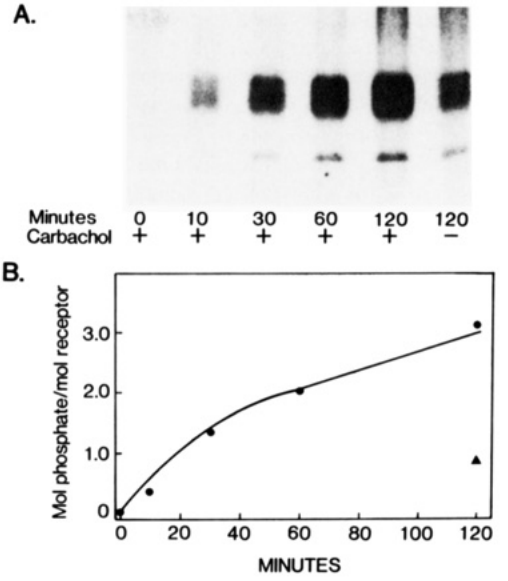

FIGURE 2: Rate and extent of phosphorylation of the purified chick heart muscarinic receptor by the β-adrenergic receptor kinase. Panel A shows an autoradiogram depicting the time course of phosphorylation of mAChR by β-AR kinase. Phosphorylation was carried out with 1.8 pmol of mAChR and 1 mM carbachol in a reaction volume of 0.25 mL. Aliquots of ~0.3 pmol of receptor were removed at the times indicated and analyzed for phosphorylation by electrophoresis and autoradiography. The final lane shows phosphorylation of the receptor in reactions carried out for 120 min in the absence of added agonist. Phosphorylation observed under this condition may have been caused by agonist carried over to the phosphorylation reaction from the reconstitution procedure, as other experiments showed that low levels of phosphorylation observed in the absence of added agonist could be prevented by inclusion of the antagonist atropine. Panel B shows the rate and extent of phosphorylation of mAChR by β-AR kinase as determined from counting excised gel pices containing the receptors as shown in panel A. The triangle shows the amount of phosphorylation obtained in the absence of carbachol.

heparin also is known to inhibit casein kinase II and the low-density lipoprotein receptor kinase (Maenpaa, 1977; Kishimoto et al., 1987), heparin is not known to inhibit cAMP-dependent protein kianse or protein kinase C. Taken together, the results establish that β-AR kinase specifically recognizes as a substrate the agonist-occupied mAChR; occupancy of the receptors with an antagonist or a weak partial agonist is insufficient to allow for β-AR kinase-induced phosphorylation of the receptor. The results strongly suggest that agonists induce a specific conformational change that allows for phosphorylation of the mAChR by β-AR kinase.

In order to more accurately determine the effectiveness of the mAChR as a substrate for β-AR kinase, other properties of mAChR phosphorylation by β-AR kinase were ascertained. The rate of phosphorylation of the mAChR by β-AR kinase was determined and found to be almost identical with the rates of in vitro phosphorylation of the adrenergic receptors by β-AR kinase [Figure 2; compare to Figure 2 in Benovic et al. (1987a)]. In addition, the stoichiometry of the agonist-dependent phosphorylation of the mAChR was calculated and found to be approximately 3–4 mol of phosphate/mol of receptor under the conditions utilized (Figure 2). This stoichiometry was very similar to what was observed by us for the agonist-mediated phosphorylation of mAChR in intact cardiac cells (Kwatra & Hosey, 1986) and is also similar to the extent of phosphorylation of the purified β_2- and α_2-adrenergic receptors by β-AR kinase in vitro (Benovic et al., 1987a,b). In calculating the stoichiometry of the in vitro phosphorylation of mAChR catalyzed by β-AR kinase, we made no correction for the possible lack of phosphorylation of mAChR whose

phosphorylation sites may have been oriented on the inner side of the phospholipid vesicles and thus inaccessible to β-AR kinase. If the receptors were randomly inserted into the vesicles, as would appear to be the case for the β_2- and α_2-adrenergic receptors, then the possibility exists that half of the receptor phosphorylation sites may have been inaccessible to β-AR kinase. Thus, the true stoichiometry might approach 6–8 mol of phosphate/mol of receptor, as is the case for the β_2- and α_2-adrenergic receptors.

As a further test of the similarity of the in vitro catalyzed phosphorylation of mAChR by β-AR kinase and that observed in response to agonists in intact cells, we determined the phosphoamino acid composition of the phosphorylated receptor. After phosphorylation of the mAChR in vitro with β-AR kinase, ~70% of the acid-stable ^{32}P was recovered as phosphoserine and ~30% as phosphothreonine. This pattern was identical with what we previously reported for the phosphoamino acid content of receptors phosphorylated in intact cells in response to agonist stimulation (Kwatra et al., 1987).

The present results demonstrate that the mAChR purified from chick heart ventricles is an excellent substrate in vitro for the β-adrenergic receptor kinase. The chick heart mAChR is comparable to β_2- and α_2-adrenergic receptors (Benovic et al., 1987a) in its ability to serve as a substrate for this novel enzyme. Importantly, many of the characteristics of the in vitro phosphorylation of the mAChR by β-AR kinase are similar to the properties of agonist-induced phosphorylation of these receptors in intact cardiac cells (Kwatra & Hosey, 1986; Kwatra et al., 1987). In this regard, the extent of phosphorylation of the receptor and its phosphoamino acid composition after in vitro phosphorylation by β-AR kinase are very similar to, if not identical with, what is observed for agonist-induced phosphorylation of the chick heart mAChR in intact cells. On the other hand, the rate of phosphorylation of the mAChR by β-AR kinase in vitro, while comparable to the rates observed for the in vitro phosphorylation of adrenergic receptors by β-AR kinase (Benovic et al., 1987a), is slow compared to what has been observed for agonist-induced phosphorylation in situ (Kwatra et al., 1987). This may be due to the fact that the purified β-AR kinase has a low specific activity compared to other purified protein kinases (nmol min^{-1} mg^{-1} vs μmol min^{-1} mg^{-1}), and as such, the conditions used to demonstrate in vitro phosphorylation with β-AR kinase may be less than optimal. In addition, the pure receptors are available in low quantities for in vitro phosphorylation studies, and the use of low concentrations of receptors may also contribute to the slow rates observed. Nevertheless, the specificity of the reaction is demonstrated by the striking dependence of the phosphorylation on the agonist occupancy of the receptors. This is noteworthy since previous studies of the in situ phosphorylation of mAChR strongly suggested that a receptor-specific kinase and/or agonist-occupied receptors were necessary for the agonist-induced phosphorylation to occur (Kwatra et al., 1987). Interestingly, the chick heart mAChR can be phosphorylated in an agonist-dependent manner by a β-AR kinase like activity that is present in chick heart extracts and behaves on DEAE-Sephadex (Benovic et al., 1987b) like β-AR kinase (Kwatra and Hosey, unpublished observations). Thus, it will be interesting in future studies to determine if β-AR kinase, or a β-AR kinase like enzyme, is responsible for the agonist-mediated phosphorylation of mAChR in intact cells.

In addition to lending insights into the mechanisms possibly involved in the agonist-induced phosphorylation of mAChR, the present results extend our knowledge of the properties of β-AR kinase. The results are the first to directly demonstrate that this enzyme is capable of specifically and stoichiometrically phosphorylating a nonadrenergic receptor, in a manner similar to that previously documented for adrenergic receptors (Benovic et al., 1986, 1987a). As the chick heart mAChR is known to couple to attenuation of adenylyl cyclase, the results support the possibility (Strasser et al., 1986; Benovic et al., 1987a; Mayor et al., 1987) that this novel protein kinase may play a widespread role in the regulation of receptors coupled to adenylyl cyclase. Further studies should more clearly establish the exact role of this enzyme in receptor regulation.

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Mutation of Glycine 49 to Valine in the α Subunit of G_s Results in the Constitutive Elevation of Cyclic AMP Synthesis†

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ABSTRACT: The G-protein G_s couples hormone-activated receptors with adenylyl cyclase and stimulates increased cyclic AMP synthesis. Transient expression in COS-1 cells of cDNAs coding for the G_s α-subunit (α_s) or α_s cDNAs having single amino acid mutations Gly49 → Val or Gly225 → Thr elevated cyclic AMP levels, resulting in the activation of cyclic AMP dependent protein kinase. Stable expression in Chinese hamster ovary cells of α_sVal49 cDNA resulted in a small constitutive elevation of cyclic AMP that was sufficient to persistently activate cyclic AMP dependent protein kinase activity 1.5–2-fold over basal activity. Stable expression of wild-type α_s or α_sThr225 in Chinese hamster ovary cells was less effective in sustaining elevated cyclic AMP synthesis and kinase activation compared to α_sVal49.

Binding of GTP to the α-subunit of G-proteins regulates the activity of specific enzymes and ion channels (Gilman, 1987; Yatani et al., 1987; Neer & Clapham, 1988). Within the family of G-proteins, G_s is involved in the activation of adenylyl cyclase, resulting in the stimulation of cyclic AMP synthesis (Gilman, 1987). The sequences comprising the guanine nucleotide binding site appear to be highly conserved within the family of G-proteins (Masters et al., 1986), elongation factor Tu, and the *ras* proteins (Halliday, 1984; Leberman & Egner, 1984). Extensive genetic analysis of p21 *ras* has defined several mutations that confer transformation potential to the *ras* protein (Gibbs et al., 1985). The most common transforming mutations such as Gly12 → Val and Ala59 → Thr inhibit the intrinsic GTPase activity (McGrath et al., 1984; Gibbs et al., 1984). Other mutations in *ras* alter the guanine nucleotide binding properties of the protein (Sigal et al., 1986; Walter et al., 1986). In α_s, Gly49 and Gly225 correspond to Gly12 and Ala59, respectively, in the *ras* protein. Mutation of α_sGly49 → Val and transient expression in COS-1 cells resulted in significantly greater stimulation of cyclic AMP synthesis than expression of the wild-type α_s protein. Stable expression in Chinese hamster ovary cells of α_sVal49 resulted in a small but sufficiently elevated cyclic AMP level to constitutively activate cyclic AMP dependent protein kinase activity. The α_sThr225 mutation was significantly less effective than the α_sVal49 mutation in activating adenylyl cyclase in both transient and stable transfection assays.

Experimental Procedures

Cell Culture and DNA-Mediated Gene Transfer. Chinese hamster ovary cells (CHO K1) were maintained in F12 medium supplemented with 10% fetal calf serum. CHO K1 cells were transfected by using the protoplast fusion technique of Sandri-Goldin et al. (1981), and 24 h after transfection, the cells were placed in medium containing 500 μg/mL G418. Approximately 2 weeks after transfection, clones were isolated by using glass cloning rings and expanded and tested for stable expression of plasmid-expressed α_s constructs. Positive clones were subsequently subcloned at least one time. COS-1 cells were maintained in Dulbecco's modified Eagle's medium and 10% fetal calf serum. Expression of α_s constructs in COS-1 cells, which express large T antigen for transient plasmid amplification (Gluzman, 1981), was performed according to the DEAE-dextran procedure described by Ausubel et al. (1987). Transfected cells were screened 65–80 h after transfection.

Construction of Expression Plasmids. Expression vector pCW1-neo is a pUC13 derivative containing the SV40 enhancer, replication origin and early promoter and the SV40 splicing and polyadenylation sequence. Insertion into a unique

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