An Acidic Motif within the Third Intracellular Loop of the α_2C2 Adrenergic Receptor Is Required for Agonist-Promoted Phosphorylation and Desensitization†

Elizabeth A. Jewell-Motz‡ and Stephen B. Liggett*,‡,§

Departments of Medicine (Pulmonary), Molecular Genetics, and Pharmacology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, P.O. Box 670564, Room 7511, Cincinnati, Ohio 45267-0564

Received March 31, 1995; Revised Manuscript Received July 10, 1995⊗

ABSTRACT: The α_2C2 adrenergic receptor contains a highly acidic stretch of amino acids (EDEAEEEEEEEEEEE) within the third intracellular loop. To investigate the role of this region, we utilized site-directed mutagenesis to delete these 16 amino acids as well as to substitute them with glutamine, thereby conserving size but not charge. The wild-type and mutated α_2C2 receptors were permanently expressed in CHO cells. Neither substitution nor deletion of this region affected receptor expression, agonist or antagonist binding affinities, guanine nucleotide-sensitive formation of the high-affinity agonist−receptor−G protein complex, or functional coupling of the receptor to G_i. We considered that since α_2C2 agonist-promoted desensitization is due to phosphorylation by the β-adrenergic receptor kinase (or a related kinase), that this region may be important for establishing the acidic mileau required by this kinase. Therefore, the consequences of 30 min of agonist preexposure on subsequent α_2C2-mediated inhibition of adenylyl cyclase and on high-affinity agonist binding were determined for the wild-type and these two mutants. The wild-type α_2C2 receptor underwent ~52% functional desensitization and a ~40% loss of high-affinity binding after such exposure. In contrast, deletion and substitution of this acidic stretch of amino acids ablated desensitization as assessed by both approaches. These results correlated with those obtained in whole cell phosphorylation experiments. Cells expressing each receptor were incubated with [^{32}P]orthophosphate and exposed to agonist, and receptors were purified by immunoprecipitation. The deletion and the substitution mutant receptors underwent agonist-promoted phosphorylation at levels of only 44 ± 5% and 50 ± 15%, respectively, relative to wild-type α_2C2. Therefore, this unique acidic motif in the α_2C2 receptor is necessary for full phosphorylation induced by agonist which, in turn, is required for agonist-promoted desensitization.

Adrenergic receptors (ARs)[1] are cell-surface proteins that function as the receptors for the endogenous catecholamines epinephrine and norepinephrine. The human α_2ARs consist of three subtypes which have been cloned and characterized (Kobilka et al., 1987; Lomasney et al., 1994; Regan et al., 1988; Eason et al., 1992, 1994; Eason & Liggett, 1992; Liggett et al., 1992; Kurose & Lefkowitz, 1994). Each couples to the inhibitory guanine nucleotide binding protein, G_i, transducing signals such as inhibition of adenylyl cyclase (Liggett & Raymond, 1993) and opening of K^+ and Ca^{2+} channels (Limbird, 1988; Limbird & Sweatt, 1985). The three human subtypes, denoted here as α_2C10, α_2C4, and α_2C2 (based on the chromosomal location of the gene), exhibit high degrees of primary structural identity in the transmembrane spanning domains as compared to other regions such as the intracellular loops (Lomasney et al., 1991). The α_2ARs are unique among the adrenergic receptors in that the third intracellular loop is relatively large and subserves a number of different functions. For α_2C10, this region has been shown to be involved in coupling to G proteins (Kobilka et al., 1988; Liggett et al., 1991) and to contain sites for phosphorylation by the β-adrenergic receptor kinase (βARK) during short-term agonist-promoted desensitization (Liggett et al., 1992; Eason et al., 1995). One of the most divergent regions of primary structure of α_2C2, as compared to the other subtypes, is an extremely acidic stretch of amino acids, EDEAEEEEEEEEEEE, in the third intracellular loop. The mouse (Chruscinski et al., 1992) and rat (Zeng et al., 1994) homologues also possess similar sequences.

To investigate the structural importance of this domain, site-directed mutagenesis was used to delete this region as well as to substitute the 16 amino acids with conservative, nonacidic (glutamine) residues. These mutated receptors were then expressed in Chinese hamster ovary cells and their functional and regulatory properties ascertained.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Construction of Expression Vectors. A *Sac*I/*Kpn*I cassette derived from α_2C2 in pBC12BI was placed into M13mp19 using standard subcloning techniques. An oligonucleotide-directed site-specific mutagenesis technique (Kunkel, 1985) was used to delete nucleotides corresponding to amino acids 294−309 as well as to simultaneously delete the same 16 amino acids and

† Supported by NIH Grants HL53436 and HL41496 (S.B.L.) and HL07382 (E.A.J.-M.).

* Corresponding author. Phone: 513-558-4831. FAX: 513-558-0839.

‡ Department of Medicine (Pulmonary).

§ Departments of Molecular Genetics and Pharmacology.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: AR, adrenergic receptor; α_2AR α_2-adrenergic receptor; α_2C10, α_2C4, α_2C2, α_2AR subtypes localized to human chromosomes 10, 4, and 2, respectively; G_i, inhibitory guanine nucleotide binding protein; G_s, stimulatory guanine nucleotide binding protein; CHO cells, Chinese hamster ovary cells; βARK, β-adrenergic receptor kinase; PBS, phosphate-buffered saline.

0006-2960/95/0434-11946$09.00/0

introduce glutamine residues in their place (Figure 1). Following identification of the desired mutation using single-nucleotide tracking, the entire cassette was sequenced and subcloned into the context of the α_2C2 cDNA contained in the pBC12BI expression vector. Final constructs were analyzed by restriction analysis as well as by sequencing across the mutation site to confirm the presence of the desired mutation.

Tissue Culture, Transfection, and Membrane Preparation. Chinese hamster ovary (CHO) cells were maintained essentially as described (Eason et al., 1994). A calcium phosphate precipitation method for transfection was used to cotransfect wild-type or mutated α_2C2 constructs and pSV2neo at a ratio of 10:1 μg of plasmid DNA. Selection was performed in 1 mg/mL G418, and clonal cell lines were screened for receptor expression using [^{3}H]yohimbine binding which is described below. Cells were routinely studied at 95% confluency. When membranes were required, cells were washed with PBS and lysed in 5 mM Tris-HCl (pH 7.4), 2 mM EDTA buffer (lysis buffer) and mechanically detached by scraping with a rubber policeman. Particulates were then centrifuged at 40000g at 4 °C for 10 min. These were then further processed and resuspended in the appropriate buffers for the assays as described below.

Radioligand Binding Assays. For radioligand competition assays, particulates were resuspended in lysis buffer, homogenized with a Polytron (Brinkmann) for three 5 s bursts at 80% maximal, and then pelleted by centrifugation at 40000g at 4 °C for 10 min. Membranes were then resuspended in 50 mM Tris-HCl (pH 7.40), 0.5 mM EDTA, and 10 mM $MgSO_4$ and added to a reaction containing 100 μM GTP, 1 mM ascorbic acid, 6 nM [^{3}H]yohimbine (the concentration equal to the K_d for the wild-type and mutant α_2C2 receptors), and varying concentrations of epinephrine or phentolamine. In order to determine high- and low-affinity binding constants for epinephrine, assays were performed exactly as described above except for the absence of GTP. To delineate changes in high- and low-affinity binding parameters induced by agonist preexposure, cells in monolayers were incubated in media (without calf serum) with 0.1 mM ascorbic acid alone or ascorbic acid with 100 μM epinephrine for 30 min at 37 °C. Cells were then washed 5 times with cold PBS, membranes prepared, and competition studies carried out as described above. For saturation binding studies, membranes were resuspended in 25 mM glycylglycine, 12.5 mM $MgCl_2$, pH 7.4, and 0.5−25 nM [^{3}H]-yohimbine. Nonspecific binding was defined by incubations in duplicate tubes in the presence of 100 μM phentolamine. All reactions were carried out at 25 °C for 30 min. Agonist-promoted down-regulation of receptor expression was determined by incubating cells with 100 μM epinephrine for 24 h and determining receptor density in membranes using [^{3}H]yohimbine binding. For sequestration studies, whole cell radioligand binding was carried out at 4 °C as described (Eason & Liggett, 1992) after exposure of the cells to media alone or media with 100 μM epinephrine for 30 min. For all of the above assays, reactions were terminated by dilution in cold 10 mM Tris-HCl, pH 7.4, followed by rapid filtration over Whatman glass fiber filters. Filters were then counted in the presence of a xylene-based scintillation cocktail in a liquid scintillation counter.

Adenylyl Cyclase Assays and Short-Term Desensitization. α_2C2-mediated inhibition of adenylyl cyclase activity was determined as described (Eason et al., 1992, 1995). Because α_2C2 weakly couples to G_s, which results in increases in adenylyl cyclase at high agonist concentrations in the assay, cells were first treated with 20 μg/mL cholera toxin to ablate α_2C2−G_s coupling as described (Eason et al., 1992). For agonist-promoted desensitization, cells in monolayers were treated with media alone or media plus UK14304 (10 μM final concentration) for 30 min, placed on ice, and washed 6 times with cold PBS. Membranes were then prepared and adenylyl cyclase activities determined by methods as described elsewhere (Eason et al., 1992, 1995). Briefly, membranes were resuspended in a buffer which provided for 16 mM HEPES, 0.32 mM EDTA, and 0.64 mM $MgCl_2$, pH 7.4, as final concentrations in the assay. Incubations were carried out in (final concentrations) 2.7 mM phosphoenolpyruvate, 50 nM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 μg/mL myokinase, 0.05 mM ascorbic acid, and 1.0 μCi [α^{32}P]ATP for 45 min at 37 °C. Reactions were terminated by adding ice-cold stop solution composed of [^{3}H]cAMP and excess ATP and cAMP. Sequential chromatography over dowex and alumina columns (Salomon et al., 1974) was used to isolate [^{32}P]cAMP generated during the assay. [^{3}H]cAMP in the stop solution was used to quantitate the column recovery of cAMP. Adenylyl cyclase activities were determined in the absence (basal) and presence of 1 μM forskolin, and in the presence of 1 μM forskolin and various concentrations of α_2AR agonists as indicated.

Phosphorylation and Immunoprecipitation. Cells expressing equal levels of wild-type or mutant α_2C2 receptor were grown to ~95% confluence and incubated with [^{32}P]-orthophosphate (2.4 mCi/150 cm^2 plate) for 2 h at 37 °C in a 95% air, 5% CO_2 atmosphere. Cells were then incubated in the presence (10 μM UK14304) or absence of agonist for 20 min, washed 5 times with ice-cold PBS, and scraped in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA, 10 mM NaF, and 10 mM sodium pyrophosphate buffer. This and all subsequent steps included the protease inhibitors benzamidine (10 μg/mL), soybean trypsin inhibitor (10 μg/mL), and leupeptin (5 μg/mL). Particulates were then centrifuged at 40000g for 10 min at 4 °C. The resulting pellet was resuspended and sonicated for 15 s. α_2C2 receptors were purified by immunoprecipitation essentially as described previously for α_2C10 (Liggett et al., 1992; Eason et al., 1995) using polyclonal antisera directed against amino acids 211−340 of the third intracellular loop of α_2C2 (Kurose et al., 1993; Kurose & Lefkowitz, 1994). This antisera recognizes both agonist and nonagonist occupied forms of α_2C2 with equal efficiency (Kurose & Lefkowitz, 1994). Solubilization of the membrane fraction was carried out in PBS containing a final concentration of 1% Triton X-100, 0.05% SDS, 1 mM EDTA, and 1 mM EGTA by gently stirring at 4 °C for 2 h. Unsolubilized material was then removed by centrifugation at 40000g at 4 °C for 20 min. The solubilized material was then incubated with preimmune serum and protein A−Sepharose beads for 30 min at ambient temperature to remove nonspecific immunoprecipitant. After brief centrifugation, the supernatant was removed and the receptor immunoprecipitated by incubation with the α_2-C2 antibody (1:200) and protein A−Sepharose beads for 16 h at 4 °C. The beads were then washed 5 times by centrifugation and resuspension, sonicated in SDS sample

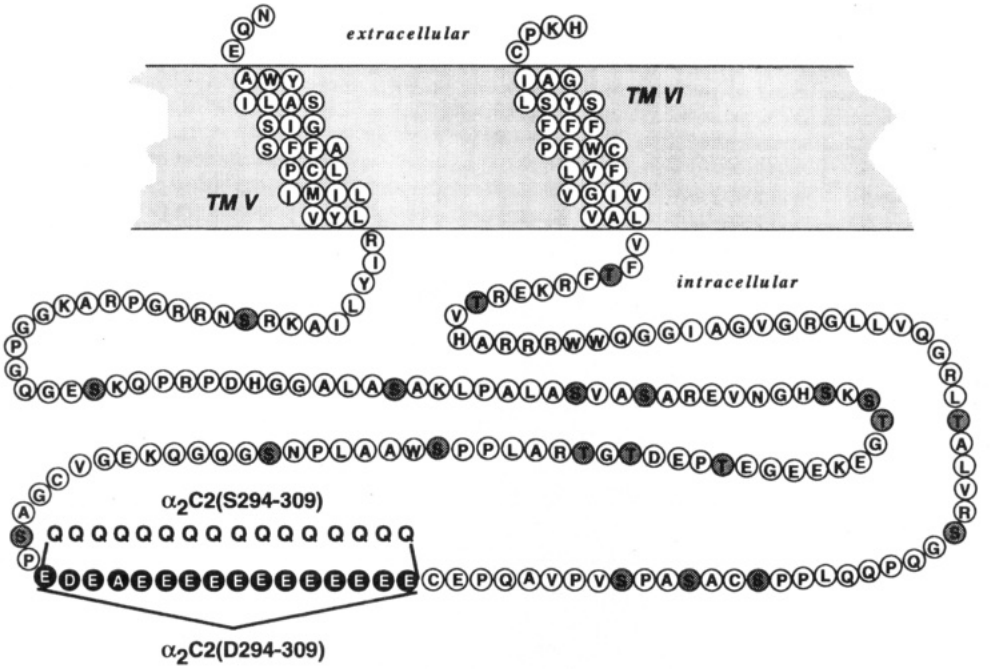

FIGURE 1: Amino acid sequence of transmembrane-spanning domains V and VI and the third intracellular loop of the α_2C2 adrenergic receptor. Black circles indicate the highly acidic stretch of amino acids localized in the third intracellular loop. The alterations introduced into this region are indicated above and below the black circles. α_2C2(S294−309) represents the substitution of this region with glutamine (Q), and α_2C2(D294−309) represents the deletion of these amino acids. Also highlighted (gray circles) are 21 serine or threonine residues which represent candidate amino acids for phosphorylation by βARK.

buffer, and removed by brief centrifugation. Proteins contained in the supernatant were then fractionated on a 10% SDS−polyacrylamide gel. Equal amounts of receptor were loaded in each lane. Autoradiography was used to detect phosphorylated α_2C2 receptor, and the amount of radioactivity on the gel was quantitated on a Molecular Dynamics PhosphorImager with ImageQuant Software. For presentation purposes, autoradiograms were produced by exposing the gels to X-ray film for ~16 h.

Western Analysis. Equal amounts of receptor proteins contained in the membrane fractions (prepared as described with the aforementioned protease inhibitors) isolated from CHO cells expressing the wild-type α_2C2, the deletion mutant, or the substitution mutant were fractionated on a 10% SDS−polyacrylamide gel and electroblotted onto nitrocellulose at 400 mA for 30 min using a Milliblot-SDE Transfer System (Millipore). The Western blot was performed by blocking the filter with 5% nonfat dry milk and then incubating with a 1:6000 dilution of the α_2C2 antisera. This was followed by incubation with an anti-rabbit horseradish peroxidase conjugated second antibody, and the blots were developed using enhanced chemiluminescence (DuPont−New England Nuclear).

Data Analysis. Data from radioligand competition and saturation binding studies, as well as adenylyl cyclase dose−response studies, were analyzed by iterative, nonlinear least-squares regression (Eason & Liggett, 1992). The extent of agonist-promoted phosphorylation was calculated by subtracting the phosphorimager counts of non-agonist-exposed receptor from those of agonist-exposed receptor, thus yielding net receptor phosphorylation induced by agonist. For the wild-type α_2C2, this was set at 100%. Mutant receptor phosphorylations from the same experiment were determined similarly and reported as a percentage of wild-type. This approach has been utilized by us (Liggett et al., 1989; Eason et al., 1995) and others (Hausdorff et al., 1989) and provides for more consistent results than expressing the data as fold-stimulation over basal. Comparisons were by two-tailed paired or unpaired Student's *t*-tests, as appropriate, with significance considered at $p < 0.05$.

Materials. Molecular biological reagents were purchased from New England Biolabs, and tissue culture supplies were from JRH Biosciences. Radioisotopes were from DuPont−New England Nuclear. All other reagents were purchased from sources listed previously (Eason et al., 1992; Eason & Liggett, 1992).

RESULTS

Our objective in this study was to investigate the role of a unique acidic region (EDEAEEEEEEEEEEE) localized in the intracellular portion of the α_2C2 as it relates to receptor function. Site-directed mutagenesis was used to delete this region as well as to substitute the 16 amino acids with glutamine residues. Figure 1 highlights this region of α_2C2 and also illustrates the two mutated receptors. As can be seen, this highly acidic motif is located within the third intracellular loop, a region known to contain critical determinants for both receptor coupling to G-proteins (Kobilka et al., 1988; Liggett et al., 1991) and short-term agonist-promoted desentization (Liggett et al., 1992; Eason et al., 1995) for α_2C10. The latter has been shown to be primarily due to receptor phosphorylation of serine residues by βARK (Liggett et al., 1992; Kurose & Lefkowitz, 1994; Eason et al., 1995). As is shown, there are 21 serine or threonine residues in the third intracellular loop, some of which are in close proximity to the above sequence. Since recent studies have shown that βARK requires an acidic milieu (Onorato et al., 1991; Chen et al., 1993), we also considered that the aforementioned sequence might provide such an environment, and therefore play an important role during agonist-

Table 1: Pharmacological Characteristics of the Wild-Type and Mutated α_2C2 Receptors Expressed in CHO Cells[a]

	α_2C2	α_2C2(D294−309)	α_2C2(S294−309)
	Ligand Binding		
[^{3}H]yohimbine binding			
B_{max} (fmol/mg of protein)	2078 ± 114	2105 ± 118	2971 ± 265
K_d (nM)	5.5 ± 0.1	6.6 ± 0.2	5.7 ± 0.2
competition with epinephrine			
−GTP: K_L (μM)	1.4 ± 0.3	0.9 ± 0.1	1.4 ± 0.1
K_H (nM)	30.2 ± 6.7	11.6 ± 2.2*	27.1 ± 9.2
% R_H	48.9 ± 2.8	45.9 ± 3.7	32.0 ± 3.4*
+GTP: K_i (μM)	2.3 ± 0.1	2.0 ± 0.1	1.5 ± 0.1
competition with phentolamine			
K_i (nM)	22.1 ± 1.0	25.0 ± 0.7	18.4 ± 1.8
	Adenylyl Cyclase Activity (pmol min^{-1} mg^{-1})		
basal	13.9 ± 0.6	14.9 ± 0.6	9.9 ± 1.3
forskolin (1 μM)	26.6 ± 1.6	35.0 ± 1.5	24.2 ± 1.7
forskolin + UK14304 (max response)	14.1 ± 1.0	12.5 ± 0.4	13.7 ± 1.2
EC_{50} (UK14304) (μM)	3.6 ± 0.4	3.6 ± 0.3	7.6 ± 1.2

[a] Radioligand binding and adenylyl cyclase studies were conducted as described under Experimental Procedures. Shown are means ± SEM of three to five experiments. B_{max}, receptor density; K_d, equilibrium dissociation constant; K_H and K_L, high- and low-affinity dissociation constants for agonist. Asterisks, $p < 0.05$.

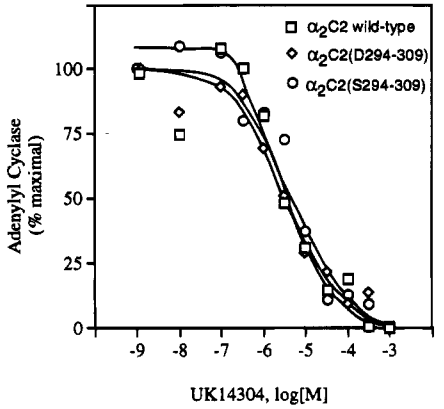

FIGURE 2: Inhibition of adenylyl cyclase activity for the wild-type and mutated α_2C2 receptors. Agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activity was determined in membranes prepared from CHO cells as described under Experimental Procedures. Shown are the mean values from 4−5 experiments. Maximal adenylyl cyclase activities (pmol min^{-1} mg^{-1}) for the wild-type α_2C2, α_2C2(D294−309), and α_2C2(S294−309) were 12.5 ± 4.3, 22.5 ± 3.5, and 10.5 ± 4.1, respectively.

promoted desensitization. The indicated mutated receptors were thus expressed in CHO cells, which we have previously found useful for characterization of α_2AR coupling and agonist-promoted desensitization.

Table 1 summarizes the pharmacologic characteristics of the wild-type and mutant receptors. Both mutated receptors bound [^{3}H]yohimbine with the same affinity as was found for wild-type α_2C2. Similarly, in competition studies performed in the presence of GTP, both receptors displayed the same affinities for the agonist epinephrine and the antagonist phentolamine as compared to wild-type. Agonist binding was found to be guanine nucleotide sensitive, in that competition studies performed in the absence of GTP revealed shallow curves which were best fit to a two-site model. The binding constants for the high- and low-affinity forms of the receptor were very similar between the mutated and wild-type α_2C2 receptors, although a few minor, but statistically significant, differences were noted (Table 1). In adenylyl cyclase studies (Table 1 and Figure 2), both mutated receptors were found to be fully functional. Maximal agonist-mediated inhibition of adenylyl cyclase was similar with the mutant receptors as compared to the wild-type. The EC_{50} for inhibition with the deletion mutant was the same as that found with the wild-type receptor and trended toward being slightly higher for the substitution mutant. Thus, the above results reveal that neither the substitution of this acidic stretch of amino acids with 16 glutamines nor the deletion of these residues significantly alters the ligand binding or functional properties of α_2C2.

Short-term agonist-promoted desensitization was studied in a manner similar to that described previously (Eason & Liggett, 1992; Eason et al., 1994, 1995). Cells were incubated with media alone or with media containing the agonist UK14304 (10 μM) for 30 min and extensively washed, membranes were prepared, and agonist-mediated inhibition of forskolin-stimulated activity was delineated. As previously shown (Eason & Liggett, 1992), desensitization of wild-type α_2C2 expressed in CHO cells is manifested by an increase in the EC_{50} for agonist-mediated inhibition of adenylyl cyclase (Table 2). For wild-type α_2C2, we observed an increase from 3.6 ± 0.4 μM to 7.5 ± 0.4 μM. As previously described, α_2AR desensitization can be further quantitated by examining adenylyl cyclase activities at a submaximal concentration of agonist (near the EC_{50}) in the assay. This identifies differences between responses when curves diverge primarily due to changes in EC_{50} (Hausdorff et al., 1989; Clark et al., 1989). Thus, wild-type α_2C2 agonist preexposure resulted in substantially less inhibition of adenylyl cyclase at this concentration (Figure 3) as compared to the untreated receptor. The higher adenylyl cyclase activities after preexposure amounted to an ~50% desensitization of receptor function for wild-type α_2C2. In marked contrast, adenylyl cyclase activities at submaximal concentrations in the assay were not different between control and agonist-treated cells for the two mutant receptors (Figure 3 and Table 2). Consistent with these results, there was no change in the EC_{50} for the deletion (3.6 ± 0.7 to 2.8 ± 0.5 μM) or the substitution (7.6 ± 2.5 to 6.7 ± 1.8 μM) mutants.

The functional desensitization observed with wild-type α_2-C2 after agonist exposure has previously been shown to be associated with a loss of high-affinity agonist binding (Eason & Liggett, 1992). Such a loss is thought to represent a decrease in formation of the high-affinity ternary complex due to receptor−G_i uncoupling initiated by receptor phos-

Table 2: Desensitization of Wild-Type and Mutated α_2C2 Expressed in CHO Cells[a]

	α_2C2		α_2C2(D294−309)		α_2C2(S294−309)	
	−agonist	+agonist	−agonist	+agonist	−agonist	+agonist
EC_{50} (μM)	3.6 ± 0.4	7.5 ± 0.4*	3.6 ± 0.3	2.8 ± 0.2	7.6 ± 1.3	6.7 ± 0.9
adenylyl cyclase act. (% forskolin at submax [UK14304])	50.2 ± 8.1	76.6 ± 2.3*	49.9 ± 2.4	39.5 ± 6.4	41.9 ± 10.5	45.1 ± 8.0
% desensitization	52.4		−20.7		7.8	

[a] Data shown are from experiments described in Figure 3. Short-term exposure (30 min) of UK14304 increased the EC_{50} for α_2C2 ~2-fold, while the EC_{50} for both the deletion mutant [α_2C2(D294−309)], and the substitution mutant [α_2C2(S294−309)] was unchanged. Desensitization was determined by examining the adenylyl cyclase activities at a submaximal concentration of UK14304 in the assay. For wild-type α_2C2 and the deletion mutant, this submaximal concentration was 3 μM. Because the basal EC_{50} for the substitution mutant was slightly higher, the adenylyl cyclase activities at the submaximal concentration of 10 μM were used for this comparison. Asterisks, $p < 0.005$.

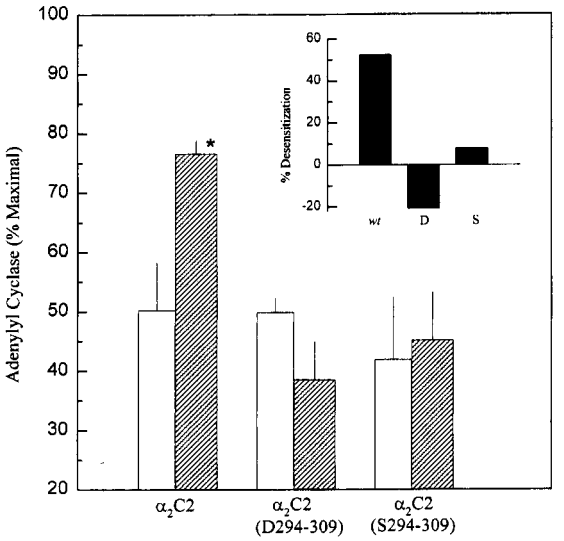

FIGURE 3: Effects of short-term agonist exposure on wild-type and mutated α_2C2 expressed in CHO cells. CHO cells separately expressing α_2C2, α_2C2(D294−309), or α_2C2(S294−309) were incubated in media alone or in media containing 10 μK UK14304 for 30 min. Adenylyl cyclase activities were determined in membranes prepared from these cells as described under Experimental Procedures. Shown are the means of adenylyl cyclase activities from 4−5 experiments with cells not exposed (open bars) or exposed (hatched bars) to agonist. Desensitization was calculated as described in Table 2. The inset illustrates the percent desensitization undergone by the wild-type α_2C2 (wt), α_2C2(D294−309) (D), and α_2C2(S294−309) (S). The comparison of adenylyl cyclase activities at submaximal [UK14304] clearly shows that while the wild-type α_2C2 undergoes desensitization, α_2C2(D294−309) and α_2C2(S294−309) do not. The EC_{50} values, the adenylyl cyclase activities at submaximal [UK14304], and the percent desensitization are summarized in Table 2.

phorylation. To corroborate the results obtained in functional assays, we conducted agonist competition studies (in the absence of guanine nucleotide) in membranes from cells expressing each receptor which had, or had not, been pretreated with agonist. These results are shown in Table 3. Agonist exposure to wild-type α_2C2 decreased the percentage of high-affinity sites (% R_H) from 48.9 ± 2.8% to 30.0 ± 3.7% ($p < 0.005$) without a change in K_H or K_L. In contrast, for the deletion and the substitution mutants, the % R_H was unaltered by agonist exposure. These results are consistent with those obtained in functional studies in that neither mutant receptor underwent agonist-promoted desensitization.

We next performed whole cell phosphorylation studies of wild-type α_2C2, the deletion, and the substitution mutant under similar conditions as those used for desensitization. For receptor purification, an antibody directed to α_2C2 was utilized for all three receptors (Kurose et al., 1993). As shown in the Western blot of Figure 4, this antibody recognized all three receptors with similar efficiencies. The wild-type α_2C2 migrated at a molecular mass of ~53 kDa, consistent with what has previously been reported (Kurose & Lefkowitz, 1994). The deletion mutant migrated at ~42 kDa and the substitution mutant at ~49 kDa. Figure 5 shows the results of a typical phosphorylation study. As can be seen in Figure 5A, wild-type α_2C2 undergoes phosphorylation during exposure to agonist for 20 min. Both mutant receptors also underwent phosphorylation, but the degree of net phosphorylation was clearly less for both mutants as compared to wild-type. For these experiments (see Figure 5B), the deletion mutant was found to undergo agonist-promoted phosphorylation of 44 ± 5% and the substitution mutant 50 ± 15% relative to wild-type α_2C2. (Comparisons of maximal receptor phosphorylation from agonist-treated cells without consideration of the "basal" levels gave similar, ~50%, reductions in phosphorylation for both mutant receptors.) Thus, both mutations significantly altered the level of agonist-promoted phosphorylation, consistent with no detectable change in physical G-protein coupling as delineated in agonist competition studies and a loss of functional desensitization as observed in adenylyl cyclase studies.

Because little is known about the relationship between α_2-AR phosphorylation and agonist-promoted sequestration and down-regulation, these parameters were assessed for the wild-type and the deletion mutant. To assess the amount of sequestration, cells were exposed to agonist for 30 min prior to assaying. For the deletion mutant, basal (19.6 ± 5.3%) and agonist-induced sequestration (39.6 ± 1.6%) was not different than observed for the wild-type α_2C2 (17.9 ± 3.2% and 31.8 ± 6.5%, respectively, $n = 3$). For down-regulation, cells were exposed to agonist for 24 h. Both the deletion mutant and the wild-type α_2C2 down-regulated receptor expression to the same extent (27.9 ± 8.8% vs 26.5 ± 3.5%, respectively, $n = 3$).

DISCUSSION

The α_2C2-adrenergic receptor contains a unique, highly acidic region (Figure 1) consisting of 16 amino acids (EDEAEEEEEEEEEEEE) in the third intracellular loop which is not present in other adrenergic receptors (including the other α_2AR subtypes) and is found rarely within the family of G protein coupled receptors. To investigate the structural importance of this domain, we constructed both a deletion mutant lacking the aforementioned sequence and a mutant which contained a stretch of glutamines substituted for this sequence. A functional alteration induced by the former, but not the latter, would suggest that size alone was

Table 3: Alterations in High-Affinity Binding during Short-Term Agonist-Promoted Desensitization[a]

	% R_H		K_H (nM)		K_L (μM)	
	C	A	C	A	C	A
α_2C2	48.9 ± 2.8	30.0 ± 3.8*	30.2 ± 6.7	22.7 ± 3.6	1.4 ± 0.3	1.05 ± 0.1
α_2C2(D294−309)	45.9 ± 3.7	45.4 ± 3.2	11.7 ± 2.2	7.43 ± 2.6	0.9 ± 0.1	0.8 ± 0.2
α_2C2(S294−309)	32.0 ± 3.4	31.4 ± 0.4	27.1 ± 9.2	23.1 ± 7.2	1.4 ± 0.1	1.0 ± 0.1

[a] Membranes were prepared from CHO cells separately expressing the wild-type and mutated α_2C2 receptors following 30 min incubation in either control media (C) or media containing agonist (A). Competition binding assays were performed in the absence of GTP as described under Experimental Procedures. The percentage of receptors in the high-affinity form (% R_H) decreased for the wild-type α_2C2, but not for α_2C2 containing either the deletion or the substitution mutations. The change in high-affinity binding for α_2C2 is consistent with the functional uncoupling observed during short-term agonist-promoted desensitization. Likewise, the lack of a change in % R_H for either of the two mutants is consistent with their inability to functionally desensitize. Asterisk, $p < 0.005$.

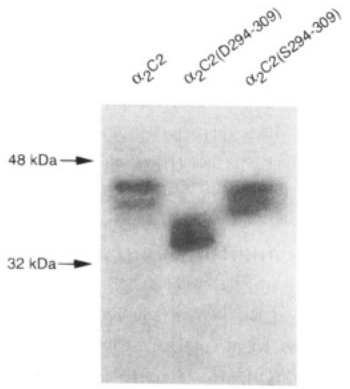

FIGURE 4: Western blots of wild-type and mutant α_2C2 receptors. Immunoblotting with the α_2C2-specific antisera was carried out using chemiluminescence as described under Experimental Procedures. The antisera identified wild-type α_2C2 at the expected molecular mass (Kurose & Lefkowitz, 1994) as well as the mutated receptors, although these were at lower molecular masses than wild-type receptor.

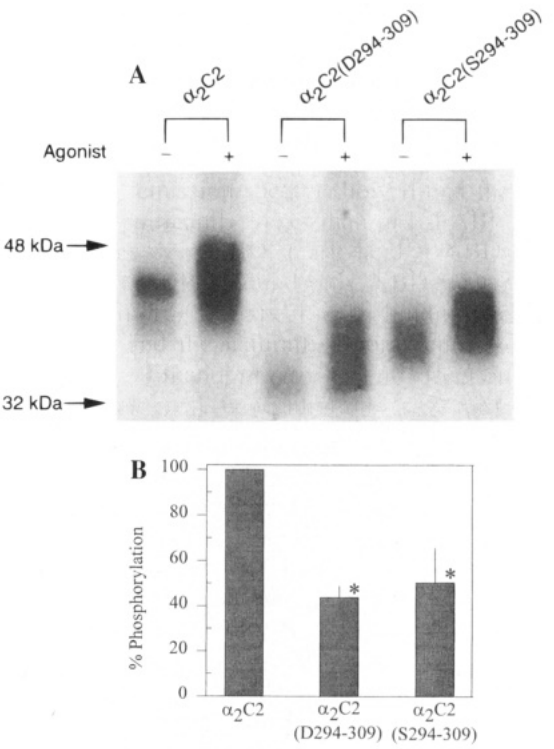

FIGURE 5: Agonist-promoted phosphorylation of wild-type and mutant α_2C2 receptors. CHO cells expressing wild-type or mutated α_2C2 were incubated with [^{32}P]P_i for 2 h, exposed to media alone (−agonist) or 10 μM UK14304 (+agonist) for 20 min at 37 °C, and then purified by immunoprecipitation using the α_2C2 antibody as described under Experimental Procedures. Panel A shows an autoradiograph of a single representative experiment, showing that agonist-promoted phosphorylation was depressed in both mutants compared to wild-type. Panel B shows the mean results from three such experiments. For these experiments, α_2C2(D294−309) and α_2C2(S294−309) underwent 44 ± 5% and 50 ± 15% phosphorylation, respectively, compared to wild-type α_2C2 ($p < 0.001$).

the primary basis for such an alteration. On the other hand, the charge (or some other specific property of these amino acids) of this region would be implicated if the substitution mutant displayed altered properties as well.

Both mutated receptors showed similar ligand binding properties for agonists and antagonists compared to wild-type α_2C2 (Table 1), which was not entirely unexpected since the domains for ligand binding lie in the membrane-spanning regions (Liggett & Raymond, 1993). It was also clear that neither mutation significantly affected G protein coupling. Each displayed formation of the high-affinity agonist−receptor−G protein ternary complex as assessed in agonist competition studies performed in the absence of guanine nucleotide. In adenylyl cyclase assays, the receptors appeared to be fully functional in that the maximal extent and the EC_{50} for agonist-mediated inhibition of forskolin-stimulated adenylyl cyclase activities were similar between the two mutated receptors and wild-type α_2C2 (Table 1 and Figure 2).

We next explored whether this motif might play a role in agonist-promoted desensitization of α_2C2. Evidence from several studies implicates phosphorylation of the receptor as the key mechanism during such desensitization, and that this phosphorylation is due to βARK or the structurally similar kinase denoted βARK2. In CHO cells expressing α_2C2, agonist exposure is accompanied by a rapid loss of high-affinity agonist binding and functional uncoupling of the receptor from G_i (Eason & Liggett, 1992)—both consistent with what has been found with βARK-mediated desensitization of β_2AR (Liggett & Lefkowitz, 1993). Second, in whole cell phosphorylation studies, α_2C2 expressed in CHO cells undergoes rapid phosphorylation during agonist exposure (Kurose & Lefkowitz, 1994). In similar studies with COS cells, α_2C2 agonist-promoted phosphorylation was found to be enhanced by coexpression with βARK (Kurose & Lefkowitz, 1994). Finally, using membranes from Sf9 cells expressing α_2C2, it has recently been shown (Pei et al., 1994) that the α_2C2 receptor is phosphorylated *in vitro* in an agonist-dependent manner by both βARK and βARK2. Studies using peptides as substrates for βARK-mediated phosphorylation in a reconstituted phospholipid vesicle system have shown that an acidic environment is

required for phosphorylation (Onorato et al., 1991; Chen et al., 1993).

Given the above, we considered that the 16 amino acid motif might provide the acidic milieu for βARK phosphorylation during α_2C2 agonist-promoted desensitization. We therefore investigated agonist-induced functional desensitization, high-affinity agonist binding, and receptor phosphorylation of α_2C2 and the two mutants. That α_2C2 undergoes short-term agonist-promoted desensitization in CHO cells has been described by two independent groups (Eason & Liggett, 1992; Kurose & Lefkowitz, 1994). In our approach, this desensitization is manifested as an increase in the EC_{50} for inhibition of adenylyl cyclase and is clearly evident when one examines the inhibition of adenylyl cyclase at a submaximal concentration of agonist in the assay. We found that such desensitization, which amounts to ~50% loss of function, was completely absent in both mutated receptors (Table 2 and Figure 3). Consistent with this lack of agonist-induced functional uncoupling found with these two mutants, neither displayed a decrease in high-affinity binding sites after agonist exposure (Table 3). On the basis of our initial hypothesis, we suspected that the loss of desensitization was due to a lack (or decrease) in agonist-promoted receptor phosphorylation. Indeed, both mutated receptors displayed only ~50% agonist-promoted phosphorylation as compared to wild-type α_2C2. This loss of receptor phosphorylation with the two mutant receptors resulted in total ablation of agonist-promoted desensitization. This suggests that full phosphorylation of this receptor may be necessary in order to evoke desensitization. The observation that partial phosphorylation leads to a complete loss of desensitization is not without precedence. We have recently observed with mutant α_2C10 receptors that partial phosphorylation was insufficient to induce desensitization (Eason et al., 1995). Mutant receptors lacking two of the four serines that are phosphorylated by βARK underwent phosphorylations of ~50% that of wild-type α_2C10 and failed to undergo any detectable desensitization. Taken together with our current results with α_2C2, it appears that there are strict requirements for the conformational change induced by βARK-mediated phosphorylation necessary for functional uncoupling.

In summary, we have assessed the importance of a highly acidic 16 amino acid portion of the third intracellular loop of the α_2C2 receptor which appears to be necessary for short-term agonist-promoted desensitization due to receptor phosphorylation. We recognize that in mutagenesis studies of this type, where a relatively long stretch of residues is altered, that losses or gains of unsuspected properties may go undetected and contribute to the mutated receptor phenotype. We have approached this potential limitation by studying two different mutated receptors and by assessing the consequences using complimentary approaches. Given the agreement of the results obtained with functional adenylyl cyclase assays, high-affinity agonist binding studies, and phosphorylation studies, we feel confident that this region plays a critical role in agonist-promoted desensitization of α_2C2. In addition, it does not appear to be important in agonist-promoted sequestration or down-regulation. It is interesting to note that at least two other G protein coupled receptors also contain this motif. One of the somatostatin receptor subtypes, denoted SSTR3, has a highly homologous region (EEEEDEEEEERREEEE) in its third intracellular loop (Yasuda et al., 1992) which is not found in any of the other somatostatin receptor subtypes. Similarly, of the five muscarinic acetylcholine receptor subtypes, the m1 receptor has a homologous sequence (EEEEEDESME) in its carboxyl terminus (Peralta et al., 1994). The importance of these regions, however, with regard to receptor function for these two receptors has not been studied. While the precise residues phosphorylated during agonist-promoted desensitization of α_2C2 are not known, studies with α_2C10 mutants (Liggett et al., 1992; Eason et al., 1995) and peptides (Onorato et al., 1991; Chen et al., 1993) point toward four serines (amino acids 296−299 in α_2C10) in the third intracellular loop, which are preceded by two glutamic acids, as being the βARK phosphorylation sites. No homologous sequence is present in α_2C2. Given the requirement for nearby acidic residues for βARK-mediated phosphorylation, our studies strongly suggest that the EDEAEEEEEEEEEEE motif in α_2C2 subserves this function. Clearly, though, such an extensive series of acidic residues is not required for βARK-mediated phosphorylation of other G protein coupled receptors. However, *within the context of the* α_2*C2*, such a motif appears to be required.

ACKNOWLEDGMENT

We thank Robert J. Lefkowitz for providing the α_2C2-specific antibody used in our studies. We also extend our appreciation to Cheryl Theiss and Elizabeth Donnelly for technical assistance and Katie Gouge for manuscript preparation.

REFERENCES

Chen, C. Y., Dion, S. B., Kim, C. M., & Benovic, J. L. (1993) *J. Biol. Chem. 268*, 7825.

Chruscinski, A. J., Link, R. E., Daunt, D. A., Barsh, G. S., & Kobilka, B. K. (1992) *Biochem. Biophys. Res. Commun. 186*, 1280.

Clark, R. B., Friedman, J., Dixon, R. A., & Strader, C. (1989) *Mol. Pharmacol. 36*, 343.

Eason, M. G., & Liggett, S. B. (1992) *J. Biol. Chem. 267*, 25473.

Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., & Liggett, S. B. (1992) *J. Biol. Chem. 267*, 15795.

Eason, M. G., Jacinto, M. T., Theiss, C. T., & Liggett, S. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11178.

Eason, M. G., Moreira, S. P., & Liggett, S. B. (1995) *J. Biol. Chem. 270*, 4681.

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., & Lefkowitz, R. J. (1989) *J. Biol. Chem. 264*, 12657.

Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, G., Lefkowitz, R. J., & Regan, J. W. (1987) *Science 238*, 650.

Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G., & Lefkowitz, R. J. (1988) *Science 240*, 1310.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Kurose, H., & Lefkowitz, R. J. (1994) *J. Biol. Chem. 269*, 10093.

Kurose, H., Arriza, J. L., & Lefkowitz, R. J. (1993) *Mol. Pharmacol. 43*, 444.

Liggett, S. B., & Lefkowitz, R. J. (1993) in *Regulation of Cellular Signal Transduction Pathways by Desensitization and Amplification* (Sibley, D., & Houslay, M., Eds.) pp 71−97, John Wiley & Sons, London.

Liggett, S. B., & Raymond, J. R. (1993) in *Catecholamines* (Bouloux, P. M., Ed.) pp 279−306, W. B. Saunders Co., London.

Liggett, S. B., Bouvier, M., O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., & Deblasi, A. (1989) *Biochem. Biophys. Res. Commun. 165*, 257.

Liggett, S. B., Caron, M. G., Lefkowitz, R. J., & Hnatowich, M. (1991) *J. Biol. Chem. 266*, 4816.

Liggett, S. B., Ostrowski, J., Chestnut, L. C., Kurose, H., Raymond, J. R., Caron, M. G., & Lefkowitz, R. J. (1992) *J. Biol. Chem. 267*, 4740.
Limbird, L. E. (1988) *FASEB J. 2*, 2686.
Limbird, L. E., & Sweatt, J. D. (1985) in *The Receptors* (Conn, P. M., Ed.) pp 281−305, Academic Press, Orlando.
Lomasney, J. W., Cotecchia, S., Lefkowitz, R. J., & Caron, M. G. (1991) *Biochim. Biophys. Acta 1095*, 127.
Lomasney, J. W., Lorenz, W., Allen, L. F., King, K., Regan, J. W., Yang-Feng, T. L., Caron, M. G., & Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5094.
Onorato, J. J., Palzewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., & Benovic, J. L. (1991) *Biochemistry 30*, 5118.
Pei, G., Tiberi, M., Caron, M., & Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3633.
Peralta, E., Ashkenazi, A., Winslow, J. W., Smith, D., Ramachandran, J., & Capon, D. (1994) *EMBO J. 6*, 3923.
Regan, J. W., Kobilka, T. S., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J., & Kobilka, B. K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6301.
Salomon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541.
Yasuda, K., Rens-Domiano, S., Breder, C. D., Law, S. F., Saper, C. B., Reisine, T., & Bell, G. I. (1992) *J. Biol. Chem. 267*, 20422.
Zeng, D., Harrison, J. K., D'Angelo, D. D., Barber, C. M., Tucker, A. L., Lu, Z., & Lynch, K. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3102.

BI950727D