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Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function

(β_2-adrenergic receptor/guanine nucleotide-binding protein/phospholipase C/constitutive activity)

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ABSTRACT **Regions of the hamster α_1-adrenergic receptor (α_1AR) that are important in GTP-binding protein (G protein)-mediated activation of phospholipase C were determined by studying the biological functions of mutant receptors constructed by recombinant DNA techniques. A chimeric receptor consisting of the β_2-adrenergic receptor (β_2AR) into which the putative third cytoplasmic loop of the α_1AR had been placed activated phosphatidylinositol metabolism as effectively as the native α_1AR, as did a truncated α_1AR lacking the last 47 residues in its cytoplasmic tail. Substitutions of β_2AR amino acid sequence in the intermediate portions of the third cytoplasmic loop of the α_1AR or at the N-terminal portion of the cytoplasmic tail caused marked decreases in receptor coupling to phospholipase C. Conservative substitutions of two residues in the C terminus of the third cytoplasmic loop ($Ala^{293} \rightarrow$ Leu, $Lys^{290} \rightarrow$ His) increased the potency of agonists for stimulating phosphatidylinositol metabolism by up to 2 orders of magnitude. These data indicate (*i*) that the regions of the α_1AR that determine coupling to phosphatidylinositol metabolism are similar to those previously shown to be involved in coupling of β_2AR to adenylate cyclase stimulation and (*ii*) that point mutations of a G-protein-coupled receptor can cause remarkable increases in sensitivity of biological response.**

The adrenergic receptors (AR) (α_1, α_2, β_1, β_2, β_3) mediate the physiological effects of epinephrine and norepinephrine via intermediacy of GTP-binding proteins (G proteins), each of which modulates a distinct intracellular effector system. The molecular cloning of the genes and/or cDNAs of the AR (1–7) as well as of several other G-protein-coupled receptors (8–12) has revealed that these proteins share a common topographical motif consisting of seven putative transmembrane regions joined by intracellular and extracellular loops. Knowledge of the primary structure of several G-protein-coupled receptors has allowed the investigation of the structural basis for different receptor functions, in particular, ligand binding and receptor coupling to distinct G proteins. Studies with chimeric α_2/β_2AR have indicated that the specificity of β_2AR coupling to adenylate cyclase stimulation via the G_s protein lies in the region including the third intracellular loop (13). Mutagenesis studies by amino acid deletion or substitution of the third intracellular loop of the β_2AR have also indicated that this cytoplasmic region is critical for productive coupling of the β_2AR to G_s (14–16). Despite the amount of information existing about the structural basis for β_2AR coupling to G_s, very little is known about the structural domains involved in the coupling of other receptors to different G protein–effector systems. Studies with chimeric muscarinic cholinergic receptors (MAchR) have indicated that a region mainly comprising the putative third intracellular loop is involved in selective coupling of M_1- and M_2AchR to their respective effector systems (17).

We have previously shown that the α_1AR cloned from DDT_1MF-2 smooth muscle cells stimulates phosphatidylinositol (PI) hydrolysis catalyzed by phospholipase C (PLC) via an as yet uncharacterized G protein (1). In this study, we have investigated the role of the putative intracellular domains of the α_1AR in mediating its coupling to PLC by constructing a variety of chimeric, truncated, and site-directed mutant α_1AR. The mutants were expressed in COS-7 cells and tested for their abilities to bind adrenergic ligands and to activate PI metabolism. Our results identify the regions in the cytoplasmic domains of the α_1AR that are most critically involved in coupling to G-protein-mediated activation of PLC.

EXPERIMENTAL PROCEDURES

Plasmids. For the construction of chimeric β_2/α_1AR, residues 228–295 of the hamster α_1AR (1) were substituted for residues 224–274 of the human β_2AR (6) by splicing the desired restriction fragments of DNA encoding the wild-type receptors with synthetic oligonucleotide adapters. For expression studies, the β_2AR and chimeric β_2/α_1AR were subcloned into the expression vector pBC12BI (18) as described (19). For construction of the α_1AR mutants, single-stranded DNA was prepared from pTZ18R (Pharmacia) containing the cDNA of the α_1AR and used for oligonucleotide-directed mutagenesis (Amersham). The identity of each mutant was confirmed by dideoxy sequencing of single- and double-stranded DNA with Sequenase (United States Biochemical). For expression studies, the expression vector $pBC\alpha_1$ (19) was digested with *Xho* I and *Apa* I and ligated to the *Xho* I–*Apa* I restriction fragments of each mutated α_1AR species to obtain pBC12BI plasmids containing the DNA for each mutated receptor.

Mammalian Cell Expression. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (100 μg/ml) and 10% fetal bovine serum (GIBCO). COS-7 cells were transfected by the DEAE-dextran method (18) with pBC12B1 containing the DNA of different receptor species, using 2 μg of DNA per 5×10^5 cells. Cells were harvested 48 hr after transfection.

Ligand Binding. Membrane preparation and ligand binding with 2-{β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl}-tetralone ([^{125}I]HEAT) for α_1AR and [^{125}I]iodocyanopindolol ([^{125}I]ICYP) (DuPont-New England Nuclear) for β_2AR binding were performed using 1 μM prazosin and 1 μM alprenolol to determine nonspecific binding, respectively (15, 19). For saturation binding analysis, [^{125}I]HEAT and [^{125}I]ICYP con-

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Abbreviations: AR, adrenergic receptor(s); G protein, GTP-binding protein; PI, phosphatidylinositol; aa, amino acid(s); PLC, phospholipase C; MAchR, muscarinic cholinergic receptor(s); [^{125}I]HEAT, 2-{β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl}tetralone; [^{125}I]ICYP, [^{125}I]iodocyanopindolol.

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centrations ranged from 10 to 500 pM. Agonist competition binding experiments used 100 pM radioligand. Data were analyzed by nonlinear least-squares regression (20).

Inositol Phosphate Determination. Cells were labeled with [^{3}H]inositol (DuPont-New England Nuclear) at 3 μCi/ml (1 μCi = 37 kBq) in DMEM supplemented with 3% fetal bovine serum. After labeling, cells were washed and incubated in phosphate-buffered saline for 30 min, followed by a 30-min incubation in phosphate-buffered saline with 20 mM LiCl. Inositol phosphates were extracted (21) and separated on AG 1-X8 columns (22). Total inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid.

RESULTS

Previous studies of the β_2AR have indicated that the putative third cytoplasmic loop is crucial in determining coupling to G_s and that much of the C-terminal tail is not required for coupling. To assess whether these properties might be common to other members of the G-protein-coupled receptor family, we examined the potential involvement of these structural domains in coupling of the α_1AR to PI hydrolysis. Initially, two constructs were made. In the first, we constructed a truncated α_1AR (T368) by inserting a stop codon after Arg^{368} (Fig. 1, Table 1), thus deleting most of the cytoplasmic tail of the receptor. With T368, the efficacy (R_{max}) and potency (EC_{50}) of norepinephrine for stimulating PI turnover, as well as the ligand-binding properties, were similar to those of the wild-type receptor (Table 1). This indicates that the C terminus of the α_1AR beyond Arg^{368} is not required for either ligand binding or effector activation, in agreement with the results obtained with the β_2AR (16).

In the second mutant, we created a chimeric β_2/α_1AR in which the entire third intracellular loop of the human β_2AR was replaced with the corresponding region of the hamster α_1AR (Fig. 1). When the β_2AR, chimeric β_2/α_1AR, and α_1AR were expressed in COS-7 cells prelabeled with [^{3}H]inositol, epinephrine was able to stimulate release of inositol phosphates in cells expressing β_2/α_1AR or α_1AR but not in cells expressing β_2AR (Fig. 2). The effect of epinephrine on β_2/α_1AR was completely blocked by propranolol (10 μM), whereas its effect was abolished by prazosin (10 μM) in cells expressing α_1AR (data not shown). The relative order of potency of agonists for inositol phosphate release mediated by β_2/α_1AR are in agreement with those for adenylate cyclase stimulation in cells expressing β_2AR (23) (EC_{50} values of isoproterenol, epinephrine, and norepinephrine were 0.14, 0.73, and 10 μM for β_2/α_1AR and 300, 1.2, and 2.2 μM for α_1AR, respectively). Ligand binding studies showed that the β_2/α_1AR specifically bound the βAR antagonist [^{125}I]ICYP with affinity similar to that of the wild-type receptor (K_d values were 30 and 53 pM for β_2/α_1AR and β_2AR, respectively). However, the expression level of β_2/α_1AR was lower than that of β_2AR (B_{max} values were 5.5 and 17.5 pmol/mg of membrane protein for β_2/α_1AR and β_2AR, respectively). Interestingly, the K_i values of isoproterenol, epinephrine, and norepinephrine for β_2/α_1AR (0.03, 0.19, and 2 μM, respectively) were 10-fold lower than for the β_2AR (0.29, 1.62, and 27 μM, respectively). These data confirm the chimeric nature of the β_2/α_1AR, which binds ligands with the classical specificity of the β_2AR but which demonstrates the effector function of an α_1AR. These data also clearly demonstrate that the third cytoplasmic loop of α_1AR confers on β_2AR the ability to activate PLC.

To further explore which sequences within the putative third intracellular loop of the α_1AR are critical for coupling to PI hydrolysis, we mutagenized selected residues within this loop. Alignment of the amino acid sequence of the third intracellular loop of the α_1AR with the corresponding sequences of other G-protein-coupled receptors reveals that these receptors share striking homology in the N- and C-terminal portions of this domain. Besides these two portions, we identified additional regions of less striking, but still significant, homology in the third intracellular loops of the α_1AR, β_2AR, and MAchR: amino acids (aa) 242–250 of the α_1AR (MKEMSNSKE) and aa 279–287 of the M_2AchR (EKESSNDST) (11); aa 252–259 of the α_1AR (TLRIHSKN)

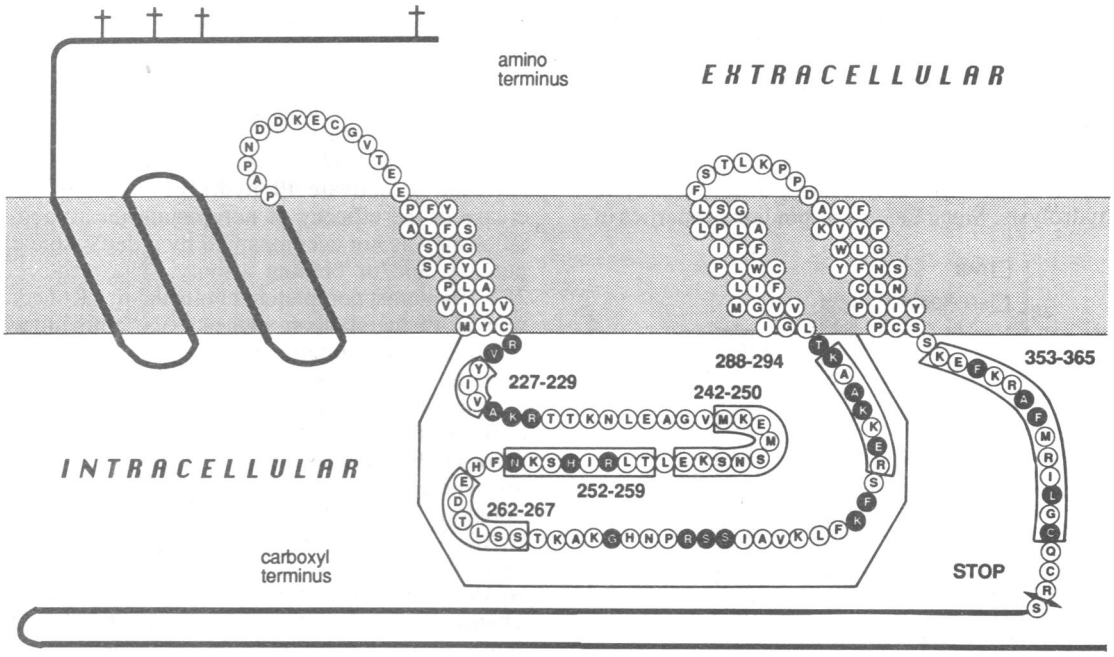

FIG. 1. Seven transmembrane-domain model of the α_1AR. The circumscribed area shows the portion of the putative third intracellular loop that was incorporated in the β_2AR to construct the chimeric β_2/α_1AR. Boxed sequences indicate the specific amino acids that were replaced in the various mutants with the corresponding sequences of the β_2AR shown in Table 1. The numbers refer to amino acid positions, and "stop" indicates the position where a stop codon was introduced to construct the truncated α_1AR T368. Solid circles indicate amino acids common to the corresponding position in the β_2AR in the regions that have been mutagenized. Potential asparagine-linked glycosylation sites near the N terminus are shown as crosses.

Table 1. Parameters of ligand binding and activation of PI metabolism obtained with mutant α_1AR

Receptor	Substitution*	Ligand binding† NE K_i, μM	Ligand binding† [^{125}I]HEAT K_d, pM	Ligand binding† [^{125}I]HEAT B_{max}, pmol/mg	NE-stimulated PI hydrolysis‡ R_{max}, % increase	NE-stimulated PI hydrolysis‡ EC_{50}, μM
α_1AR	—	15.5	68	5.7	232	3.5
S227–229	YIV → FQE	1.6	79	3.3	127	2.9
S242–250	MKEMSNSKE → LQKIDKSEG	8.5	90	3.4	127	7.5
S252–259	TLRIHSKN → EGRFHVQN	12.3	79	6.0	20	—
S262–267	EDTLSS → QVEQDG	10.7	60	4.8	156	1.5
S288–294	REKKAAK → KEHKALK	0.08	53	1.4	245	0.01
S288	R → K	9.6	65	5.5	231	2.8
S290	K → H	0.88	55	3.5	218	0.07
S293	A → L	0.20	50	1.6	200	0.02
S353–365	KEFKRAFMRILGE → PDFRIAFQELL-C	10	80	5.7	138	5.9
T368	—	5.5	63	6.0	250	3

*The locations of the α_1AR sequences that were replaced are indicated in Fig. 1.
†[^{125}I]HEAT and norepinephrine (NE) binding were measured as described under *Experimental Procedures*.
‡COS-7 cells (10^6) expressing the various receptors were stimulated with various concentrations of NE for 30 min. R_{max} indicates the percentage increase of inositol phosphates over basal induced by 100 μM NE. The results are the means of two to six independent experiments each performed in triplicate, which agreed within 20%.

and aa 237–244 of the β_2AR (EGRFHVQN) (6); and aa 262–267 of the α_1AR (EDTLSS) and aa 294–299 of the M_1AchR (EEPGSE) (11). We reasoned that these conserved sequences in the third intracellular loop might be involved in overall receptor–G protein interactions. On the other hand, the residues that differ between the α_1AR and other receptors in these conserved regions might be responsible for the specificity of α_1AR coupling to its G protein.

To test this hypothesis, we investigated whether replacement of these portions of the third intracellular loop of the α_1AR with corresponding sequences derived from the β_2AR could affect the functional properties of the resulting receptor. Table 1 shows that all the mutant receptors bound [^{125}I]HEAT with similar affinity as the α_1AR. Mutants S227–229 and S242–250, with modified sequences in the N-terminal portion of the third intracellular loop of the α_1AR, exhibited a decrease both in receptor expression levels and in the efficacy (R_{max}) of norepinephrine to promote release of inositol phosphates. The K_i and EC_{50} values of norepinephrine were similar between S242–250 and α_1AR. Interestingly, S227–229 displayed a 10-fold higher affinity for norepinephrine, but without a corresponding change in its potency to activate PI hydrolysis. Since the ligand-binding properties of these mutant receptors were comparable to those of the α_1AR, the decreased expression may reflect an impairment of processing, insertion, and/or folding of the receptor protein in the plasma membrane. These results are in agreement with previous findings showing that the regions of the third intracellular loop of the β_2AR that are adjacent to the membrane are important for normal receptor expression (24). A previous study has shown that an α_1AR-mediated increase of PI metabolism is highly dependent on the receptor number expressed in COS-7 cells (19). These observations suggest that the reduction of norepinephrine's efficacy to release inositol phosphates observed with S227–229 and S242–250 may be the result of depressed receptor expression. Therefore, the N-terminal portion of the third intracellular loop of the α_1AR does not seem to be critical for the specificity of receptor coupling to PLC, as previously proposed for β_2AR (15).

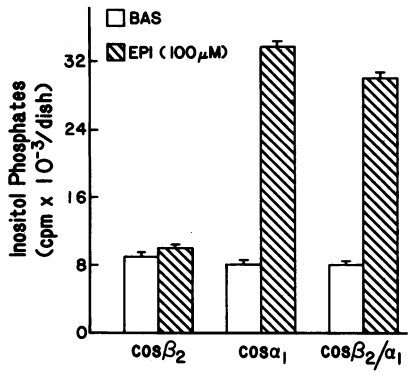

FIG. 2. Inositol phosphate release in COS-7 cells expressing β_2AR, α_1AR, and chimeric β_2/α_1AR. Total inositol phosphates were measured after a 30-min incubation in the presence of vehicle (basal, BAS) or 100 μM epinephrine (EPI). The receptor density was 15, 6.0, and 3.8 pmol/mg of membrane protein for β_2AR, α_1AR, and β_2/α_1AR, respectively. The results are means ± SEM of three independent experiments each performed in triplicate.

Substitution of aa 252–259 and 262–267 in the intermediate portion of the third intracellular loop of α_1AR did not affect receptor expression (Table 1). These substitutions, however, resulted in 90% and 32% impairment in the ability of norepinephrine to activate PI hydrolysis, respectively. The decrease in the efficacy of norepinephrine to activate PI metabolism was not accompanied by a detectable change in its potency and/or binding affinity. That replacement of aa 252–259 almost completely abolished α_1AR-mediated activation of PI metabolism suggests a prominent role of these residues in the interaction between the α_1AR and its G protein–effector system.

In addition to the third cytoplasmic loop, the N-terminal portion of the cytoplasmic tail of the β_2AR has also been shown to be implicated in the coupling of the β_2AR to G_s (15, 16). Therefore, we investigated the role of this region in α_1AR coupling to PLC activation by replacing aa 353–365 of the α_1AR with the corresponding residues of the β_2AR. This substitution resulted in 40% impairment of the ability of the α_1AR to activate PI metabolism, without any significant change in receptor expression level and the affinity or potency of norepinephrine. As previously shown for β_2AR coupling to G_s (15, 16), these results suggest that the N-terminal segment of the cytoplasmic tail of the α_1AR is important for productive coupling of the α_1AR to PLC.

In contrast to substitution mutations that resulted in an impairment of α_1AR function, a conservative substitution for aa 288–294 at the C terminus of the third intracellular loop

resulted in a dramatic change in the properties of the expressed receptor. The mutant receptor showed a 100-fold higher affinity for norepinephrine and a 300-fold higher potency of norepinephrine to activate PI metabolism. The coupling efficacy of S288–294 to PLC was even greater than for the native receptor, since the amount of mutant receptor expressed was only 25% that of the wild-type receptor.

Since substitution of aa 288–294 resulted in the conservative modification of only three residues (Arg^{288}, Lys^{290}, and Ala^{293}), we investigated which of these residues was specifically responsible for the functional modifications observed with S288–294. Thus, we individually replaced Arg^{288}, Lys^{290}, or Ala^{293} with the corresponding residue of the β_2AR. An $Arg^{288} \rightarrow$ Lys mutation did not modify the ligand-binding or functional properties of the α_1AR (Table 1). On the other hand, both an $Ala^{293} \rightarrow$ Leu and a $Lys^{290} \rightarrow$ His mutation increased the affinity and potency of norepinephrine (Table 1; Fig. 3) with the $Ala^{293} \rightarrow$ Leu substitution being the most effective. Although expression of the S293 and S290 mutant receptors was, respectively, 70% and 40% lower than that of the α_1AR, the maximal stimulation of PI metabolism was comparable to that of the wild-type receptor.

The observation that replacement of Ala^{293} or Lys^{290} specifically affected agonist, but not antagonist, binding and increased both the potency and efficacy of norepinephrine for stimulating PI metabolism suggests a direct involvement of the C-terminal portion of the third intracellular loop of the α_1AR in receptor–G protein interactions. Interestingly, the basal level of inositol phosphates in COS-7 cells expressing S288–294 was twice as high as in cells expressing α_1AR (Fig. 4). Similarly, a 40% increase in inositol phosphates was observed in COS-7 cells expressing S293 and S290, but not in cells expressing S288 (Fig. 4). The inositol phosphate content of cells expressing either the wild-type or mutant α_1AR did not differ from that of nontransfected cells (data not shown).

Because of the extraordinarily high affinity of S288–294 for norepinephrine, we investigated whether the increased inositol phosphate content of COS-7 cells expressing S288–294 receptor resulted from activation of the receptor by small amounts of catecholamines that may have been present in the cell growth medium. To test this hypothesis, cells were deprived of serum for 24 hr prior to transfection, which was also performed in the absence of serum. After transfection, cells were labeled for 24 hr with [^{3}H]inositol in medium without serum. Following this treatment, COS-7 cells expressing S288–294 still exhibited a 50–100% increase in inositol phosphates as compared to cells expressing the α_1AR (data not shown). Only when cells expressing S288–294 were grown in the presence of the αAR antagonist prazosin (10 μM) or phentolamine (100 μM) did the levels of inositol phosphates observed in the absence of agonist approximate those in the cells expressing the α_1AR (data not shown). These results might indicate that serum deprivation for 48 hr did not deplete the cells of endogenous catecholamines. Thus, minute amounts of catecholamines present in the growth medium, which would ordinarily not be sufficient to activate the α_1AR, can stimulate S288–294 due to its extraordinarily high affinity for the agonist. This effect can be blocked by the antagonist. Alternatively, our findings might indicate that S288–294 is constitutively active. Replacement of Ala^{293} with leucine and Lys^{290} with histidine might favor the active (i.e., agonist-bound) conformation of the receptor, resulting in its productive coupling to PLC even in the absence of added agonist. In this model antagonists would prevent constitutive receptor–G protein coupling by stabilizing an inactive conformation of the receptor.

DISCUSSION

By constructing a chimeric β_2/α_1AR, we have demonstrated that the third intracellular loop of the α_1AR is able to confer on the β_2AR the ability to activate PI hydrolysis. This finding extends the notion, previously shown for the β_2AR and the M_1- and M_2AchR, that the putative third intracellular loop comprises the most important determinants for receptor coupling to its specific G protein. By replacing α_1AR sequences in the cytoplasmic regions with corresponding sequences of the β_2AR, we have established that sequences in the intermediate and C-terminal portions of the loop, as well as in the N-terminal segment of the cytoplasmic tail, are important for coupling of the α_1AR to PI metabolism.

These results are in general agreement with previous findings suggesting that the C-terminal portion of the third intracellular loop and the N-terminal segment of the cytoplasmic tail of the β_2AR form a binding surface for G_s (15, 16). Since the N- and C-terminal regions of the third cytoplasmic loops of G-protein-coupled receptors have been predicted to form amphipathic α-helices, the distribution of charged and

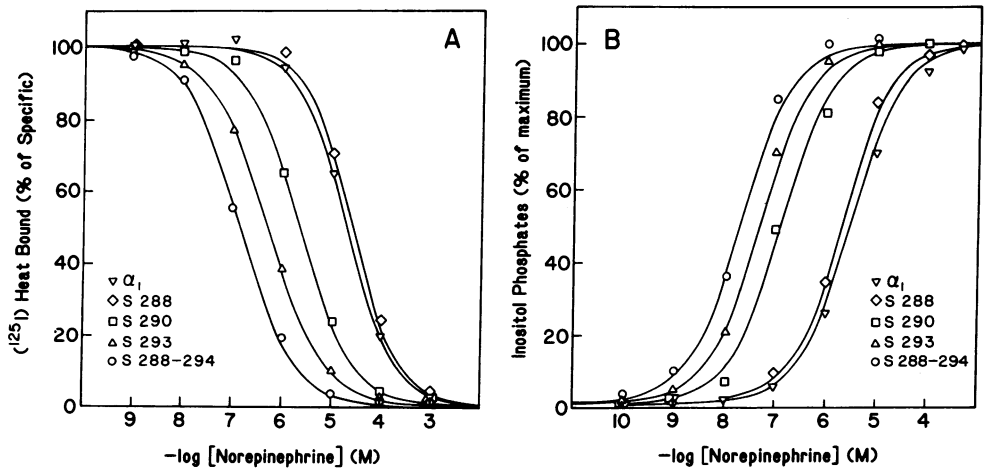

FIG. 3. Effect of conservative substitutions in the C terminus of the third intracellular loop of the α_1AR on norepinephrine binding affinity and potency to increase inositol phosphates. (*A*) [^{125}I]HEAT (100 pM) binding to COS-7 cell membranes was determined in the presence of various concentrations of norepinephrine; 100% bound was 10 pM for each membrane preparation. The K_i of norepinephrine was 9.5 μM for α_1AR, 12.5 μM for S288, 1.15 μM for S290, 0.26 μM for S293, and 0.07 μM for S288–294. (*B*) Total inositol phosphates were measured after a 30-min incubation with various concentrations of norepinephrine. The 100% maximal increase above basal did not differ significantly among cells expressing the various receptors, and ranged from 200% to 250%. The EC_{50} of norepinephrine was 3.0 μM for α_1AR, 1.2 μM for S288, 0.13 μM for S290, 0.05 μM for S293, and 0.02 μM for S288–294. Results are representative of three independent experiments.

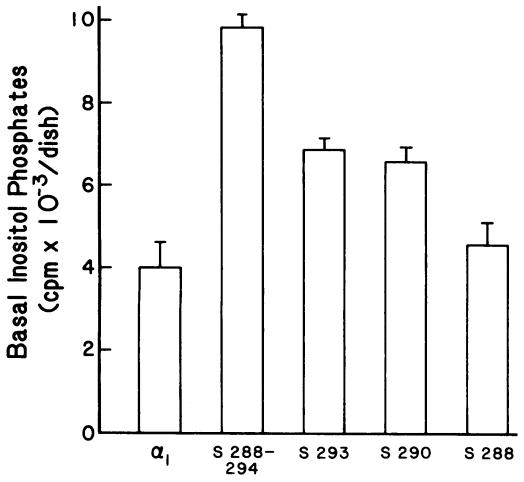

FIG. 4. Increased basal inositol phosphate content in COS-7 cells expressing mutant α_1AR with conservative substitutions in the C terminus of its third intracellular loop. Total inositol phosphates were measured in the absence of agonist stimulation. Receptor density (pmol/mg of membrane protein) was 6.2 for α_1AR, 1.2 for S288–294, 5.5 for S288, 3.0 for S290, and 1.0 for S293. Data are means ± SEM from three independent experiments each done in triplicate.

hydrophobic moments of these helices have been proposed to determine the specificity of receptor–G protein coupling (16). However, deletion of the C-terminal portion of the third intracellular loop of the β_2AR did not completely abolish β_2AR-mediated adenylate cyclase stimulation (14, 16), suggesting that other regions of the receptor are involved in coupling to G_s. In agreement with this observation is our finding that substitution of aa 252–259 in the intermediate portion of the third intracellular loop of the α_1AR almost completely abolished receptor-mediated activation of PI metabolism. It is of further interest that aa 252–259 are conserved in the third cytoplasmic loop of a novel α_1AR subtype that has been cloned recently (25), suggesting a role of this sequence as a recognition site for the G proteins coupled to the α_1AR family. In addition, our results indicate that conservative substitutions for Ala^{293} and Lys^{290} in the C-terminal portion of the third intracellular loop increased the affinity of norepinephrine binding and its potency for stimulating PI metabolism by 1–2 orders of magnitude.

Point mutations have been shown to activate several protooncogenes (26), and two single amino acid mutations constitutively activate the α subunit of G_s in a subset of human pituitary tumors (27). Our findings suggest that point mutations might activate a receptor either constitutively or by virtue of the extraordinarily high affinity of the receptor for endogenous agonist. Thus, the receptor might respond to the minute quantities of agonist circulating in the plasma even though wild-type receptor does not. Modifications of a region of the receptor implicated in G-protein coupling may represent a fertile approach to developing mutant receptors displaying activity even in the absence of agonist. Such mutants might not only help to illuminate the biochemical mechanisms involved in receptor–G protein coupling but also provide models for how point mutations might activate potentially oncogenic receptors (28).

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