Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5089–5093, July 1990
Biochemistry

Involvement of tyrosine residues located in the carboxyl tail of the human β_2-adrenergic receptor in agonist-induced down-regulation of the receptor

(hormone signaling/internalization/adenylyl cyclase/mutagenesis/isoproterenol)

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Contributed by Robert J. Lefkowitz, April 18, 1990

ABSTRACT **Chronic exposure of various cell types to adrenergic agonists leads to a decrease in cell surface β_2-adrenergic receptor (β_2AR) number. Sequestration of the receptor away from the cell surface as well as a down-regulation of the total number of cellular receptors are believed to contribute to this agonist-mediated regulation of receptor number. However, the molecular mechanisms underlying these phenomena are not well characterized. Recently, tyrosine residues located in the cytoplasmic tails of several membrane receptors, such as the low density lipoprotein and mannose-6-phosphate receptors, have been suggested as playing an important role in the agonist-induced internalization of these receptors. Accordingly, we assessed the potential role of two tyrosine residues in the carboxyl tail of the human β_2AR in agonist-induced sequestration and down-regulation of the receptor. Tyr-350 and Tyr-354 of the human β_2AR were replaced with alanine residues by site-directed mutagenesis and both wild-type and mutant β_2AR were stably expressed in transformed Chinese hamster fibroblasts. The mutation dramatically decreased the ability of the β_2AR to undergo isoproterenol-induced down-regulation. However, the substitution of Tyr-350 and Tyr-354 did not affect agonist-induced sequestration of the receptor. These results suggest that tyrosine residues in the cytoplasmic tail of human β_2AR are crucial determinants involved in its down-regulation.**

Exposure of plasma membrane-bound receptors to agonists often leads to a rapid decrease of the receptor number present at the surface of the cells. For the β_2-adrenergic receptor (β_2AR), this phenomenon is believed to play an important role in the desensitization of β-adrenergic responses that accompanies sustained stimulation (1). Two distinct processes appear to contribute to agonist-induced regulation of cell surface β_2AR density (1). The first is a rapid sequestration of the receptors away from the plasma membrane into a light vesicle fraction that leads to a reduction in the number of cell surface receptors but with no change in the total cellular receptor number. The second is a slower down-regulation of the receptors that leads to a reduction of the total cellular contingent of β_2AR. Although intracellular degradation of the receptor has been proposed to contribute to the down-regulation process (2–4), the molecular mechanisms involved in agonist-induced β_2AR sequestration and down-regulation remain largely unknown.

A growing body of indirect evidence suggests that down-regulation and/or sequestration of the β_2AR may involve its endocytosis by way of clathrin-coated vesicles (CCVs). This evidence includes the observation that several factors which perturb the internalization of receptors known to be endocytosed by way of CCVs are also found to perturb the internalization of the β_2AR. Indeed, the internalization of both the β_2AR and the epidermal growth factor receptor is inhibited by concanavilin A (5), phenylarsine oxide (6), and a reduction in the cellular ATP content (7). Moreover, high osmolarity or reduction in intracellular potassium concentration or pH, which has been shown to interfere with the formation of clathrin-coated pits (8, 9), is also found to inhibit the internalization of both the β_2AR and the transferrin receptor (31).

In recent studies, tyrosine residues located in the cytoplasmic tails of several receptors have been suggested as molecular determinants that are required for efficient and rapid agonist-induced CCV-mediated endocytosis. Indeed, mutation of specific tyrosine residues in the low density lipoprotein (10), poly-immunoglobulin (11), and mannose-6-phosphate (12) receptors greatly perturb their internalization. Moreover, the insertion of a tyrosine residue in the cytoplasmic domain of the influenza virus hemagglutinin promotes the endocytosis of this protein, which normally does not undergo internalization (13). In the present study, we have, therefore, assessed the role of two tyrosine residues located in the cytoplasmic tail of the β_2AR in agonist-induced sequestration and down-regulation of the receptor.

EXPERIMENTAL PROCEDURES

Materials. ^{125}I-labeled iodocyanopindolol, ^{125}I-labeled iodopindolol ([^{125}I]PIN), [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from New England Nuclear. (−)-Isoproterenol (Iso), (−)-alprenolol, ATP, GTP, cAMP, phospho*enol*pyruvate, myokinase, (−)-propranolol, forskolin, and isobutyl methylxanthine (IBMX) were purchased from Sigma. Pyruvate kinase was from Calbiochem. G418, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, fungizone, glutamine, trypsin, penicillin, Dulbecco's phosphate-buffered saline (PBS), and streptomycin were obtained from GIBCO. ICI-118551 and betaxolol were generously provided by Imperial Chemical Industries. CGP-12177 was generously provided by CIBA–Geigy.

Site-Directed Mutagenesis. A mutant human β_2AR was constructed, bearing alanines in place of Tyr-350 and Tyr-354 in its carboxyl tail ([Ala^{350},Ala^{354}]β_2AR), as follows. The *Eco*RI–*Hin*dIII fragment of pSPNAR (14) containing the β_2AR coding

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Abbreviations: β_2AR, β_2-adrenergic receptor; CCV, clathrin-coated vesicle; [^{125}I]PIN, ^{125}I-labeled iodopindolol; IBMX, isobutylmethylxanthine; Bt$_2$cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate; Iso, (−)-isoproterenol; [Ala^{350},Ala^{354}]β_2AR, β_2AR with Tyr-350 and Tyr-354 replaced with alanine; G_s, stimulatory guanine nucleotide-binding regulatory protein.

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sequence and ≈40 and ≈570 base pairs of 5′ and 3′ untranslated sequence, respectively, was cloned into the *Eco*RI–*Hin*dIII sites of pTZ18R (Pharmacia). Single-stranded DNA of the noncoding strand was generated using VCS-M13 helper phage (Stratagene) and served as a template for oligonucleotide-directed mutagenesis (Amersham). Identity of the mutant DNA was confirmed by dideoxynucleotide sequencing.

For eukaryotic expression, the *Eco*RI–*Hin*dIII fragment of [Ala^{350},Ala^{354}]β_2AR DNA in pTZ18R was made blunt-ended with the Klenow fragment of DNA polymerase I and cloned into the blunt-ended *Hin*dIII–*Bam*HI sites of pBC12BI (15). The *Nco* I site within the simian virus 40 origin in pBC12BI was cut, blunt-ended as above, and religated prior to insertion of the wild-type and mutant β_2AR genes; the vector was otherwise unaltered. Construction and expression of wild-type β_2AR was as described (16).

Cell Transfection and Culture. Wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR pBC12BI constructs were cotransfected with pSV2-neo (17) into transformed Chinese hamster fibroblasts (CHW-1102, hereafter called CHW) by calcium phosphate precipitation (18). Neomycin-resistant cells were selected in DMEM supplemented with 10% (vol/vol) fetal bovine serum and G418 (150 μg/ml). Resistant clones were then screened for β_2AR expression in a ^{125}I-labeled iodocyanopindolol radioligand binding assay. Cloned cell lines expressing similar numbers (i.e., ≈800 fmol/mg of membrane protein) of wild-type and mutant β_2AR were selected for further study. The transfected cells were grown as monolayers in 75-cm^2 flasks containing DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml), and glutamine (1 mM) in an atmosphere of 95% air/5% CO_2 at 37°C.

Agonist-Induced Down-Regulation and Whole-Cell Radioligand Binding Assays. Approximately 90% confluent cells were incubated at 37°C for various periods of time with DMEM supplemented as above containing either Iso alone (1 μM) or Iso (1 μM) plus N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) (1 mM) and IBMX (0.1 mM) to prevent Bt_2cAMP degradation. Control cells were incubated with medium alone. After incubation, the cells were washed twice with PBS, detached with trypsin (0.25%), washed three times with supplemented DMEM, and resuspended in PBS at a concentration of 0.1 mg of protein per ml. For radioligand binding assays, 50 μl of cell suspension was used with 0.2 μCi of [^{125}I]PIN (1 Ci = 37 GBq). Binding reactions were performed in DMEM at 13°C for 3.5 hr or at 25°C for 1.5 hr in a final volume of 0.5 ml and terminated by rapid filtration over Whatman GF/C glass fiber filters. Total receptor number was defined as the amount of radioligand binding inhibited by 0.3 μM (−)-propranolol, whereas cell surface receptor number was defined as the amount of [^{125}I]PIN binding inhibited by 0.1 μM CGP-12177.

Membrane Preparations. Cells were washed three times with 5 ml of PBS at room temperature and mechanically detached in 10 ml of ice-cold 5 mM Tris·HCl, pH 7.4/2 mM EDTA. Cells were lysed with a Polytron homogenizer (one 5-sec burst at maximum speed). The lysate was centrifuged at 45,000 × *g* for 20 min at 4°C and washed twice in the same buffer. The pelleted membranes were resuspended in 0.6 ml of a buffer containing 75 mM Tris·HCl, pH 7.4/12.5 mM $MgCl_2$/2 mM EDTA. For whole-cell and membrane preparations, protein was measured by the method of Bradford (19) using bovine serum albumin as standard.

Adenylyl Cyclase Assays. Adenylyl cyclase activities were measured by the method of Salomon *et al.* (20). Assay mixtures contained 0.02 ml of membrane suspension, 0.012 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phospho*enol*pyruvate, 0.2 unit of pyruvate kinase, 1 unit of myokinase, and 0.13 μCi of [α-^{32}P]ATP in a final volume of 0.05 ml. Enzyme activity was determined in the absence of activators (i.e., basal activity) or in the presence of 100 μM Iso, 10 μM forskolin, or 10 mM NaF. Reactions were initiated by the addition of membranes and the assay mixture incubated for 30 min at 37°C. Reactions were terminated by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (≈25,000 cpm) and cAMP was isolated by sequential chromatography on a Dowex cation-exchange resin and aluminum oxide.

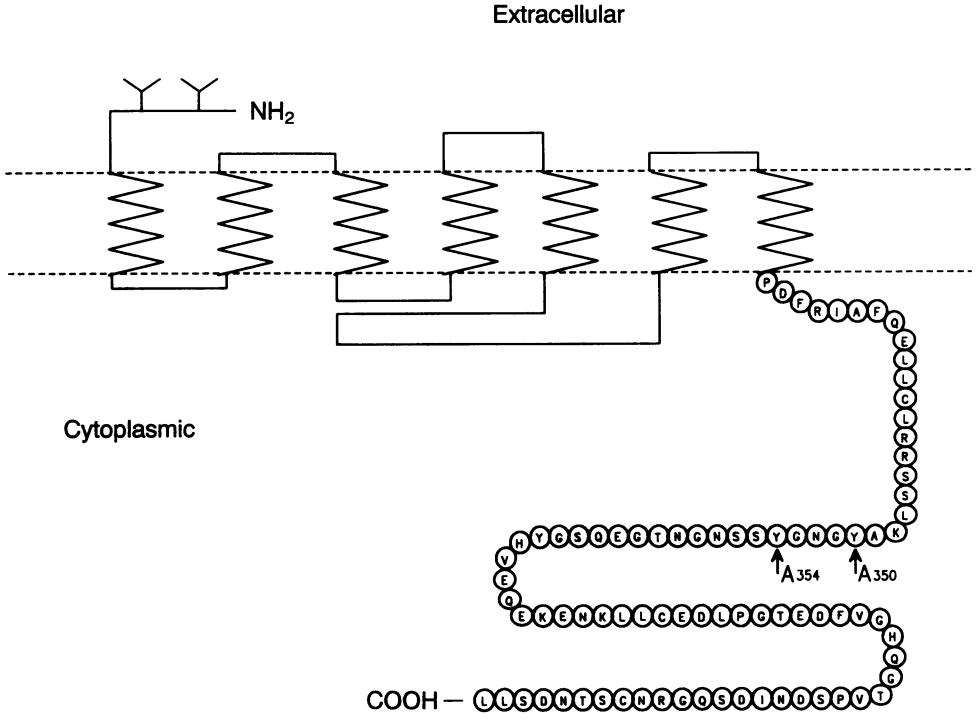

FIG. 1. Schematic representation of human β_2AR. Tyr-350 and Tyr-354 (indicated by arrows) were replaced by alanine residues to generate a mutant receptor termed [Ala^{350},Ala^{354}]β_2AR. The single-letter amino acid code is used.

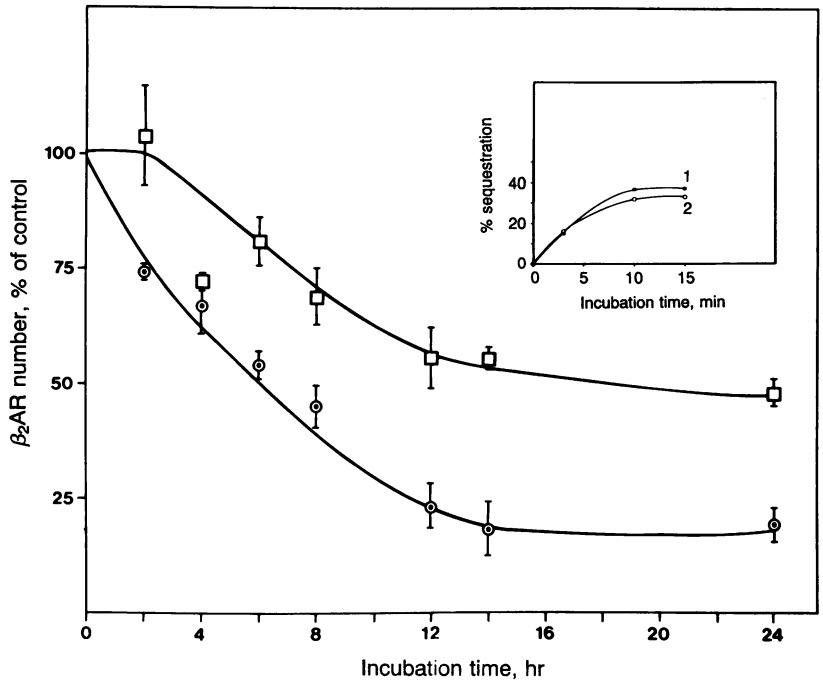

FIG. 2. Iso-induced down-regulation and sequestration of β_2AR in cells expressing wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR. Cells were incubated with 1 μM Iso for 0–24 hr at 37°C and whole-cell β_2AR number was determined by radioligand binding assay using [^{125}I]PIN as the radioligand. The number of receptors is expressed as percent of the receptor number present in untreated cells. Data are mean ± SEM of three to five determinations. ⊙, Wild-type β_2AR; □, [Ala^{350},Ala^{354}]β_2AR. (*Inset*) Sequestration of β_2AR in cells expressing wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR after incubation with 1 μM Iso for 1–15 min. Sequestration is defined as the difference between the total receptor number determined by radioligand binding using [^{125}I]PIN, and the cell surface receptor number was determined by the number of sites accessible to the hydrophilic antagonist CGP-12177. Sequestered receptor number is expressed as percent of total β_2AR number. The data shown are representative of three experiments. Curves: 1, wild-type β_2AR; 2, [Ala^{350},Ala^{354}]β_2AR.

RESULTS

A mutant human β_2AR ([Ala^{350},Ala^{354}]β_2AR) in which Tyr-350 and Tyr-354 were replaced by alanine residues was constructed by oligonucleotide-directed mutagenesis of a human β_2AR cDNA (Fig. 1). CHW cells transfected with [Ala^{350},Ala^{354}]β_2AR DNA stably expressed a functional receptor at their plasma membrane. The affinity of this mutated receptor for the β-adrenergic antagonists ^{125}I-labeled cyanopindolol, (−)-alprenolol, ICI-118551, and betaxolol was indistinguishable from that of wild-type β_2AR (data not shown). Cloned cell lines expressing similar numbers (≈800 fmol/mg of membrane protein) of wild-type and mutant receptors were selected for the study. The cellular distribution of the expressed receptor was not affected by the mutation as the proportion of [^{125}I]PIN binding sites present at the cell surface and accessible to the hydrophilic antagonist CGP-12177 was identical for wild-type and mutant receptors (78.3% ± 2.8% vs. 78.3% ± 1.2%).

Exposure of the CHW cells expressing either the wild-type β_2AR or [Ala^{350},Ala^{354}]β_2AR to the β-adrenergic agonist Iso (1 μM) led to a rapid and time-dependent reduction in the number of [^{125}I]PIN binding sites accessible to the hydrophilic antagonist CGP-12177. As shown in Fig. 2 *Inset*, this agonist treatment induces the sequestration of 35% of the total receptor number away from the cell surface, within 15 min, for both wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR. Thus, replacement of Tyr-350 and Tyr-354 of the β_2AR with alanine did not alter the pattern of agonist-induced sequestration in CHW cells. However, the time-dependent down-regulation of the total number of β_2AR induced by more prolonged exposure of the cells to Iso was significantly affected by the mutation. The decrease in specific [^{125}I]PIN binding sites was significantly slower and reached a maximum of only a 52 ± 3% decrease in cells expressing [Ala^{350},Ala^{354}]β_2AR compared with an 81 ± 4% decrease in cells expressing wild-type β_2AR (Fig. 2). It is noteworthy that despite this difference in down-regulation, the proportion of sequestered receptors was identical in cells expressing wild-type and mutant receptors even after 24 hr of agonist treatment (data not shown). Identical results were obtained in two distinct cell lines expressing [Ala^{350},Ala^{354}]β_2AR, indicating that the impairment in the down-regulation process observed is a consequence of the mutation and not an idiosyncrasy of a particular clonal cell line.

When wild-type and mutant receptors were compared in their abilities to mediate agonist stimulation of adenylyl cyclase, it was found that the mutation produced a significant

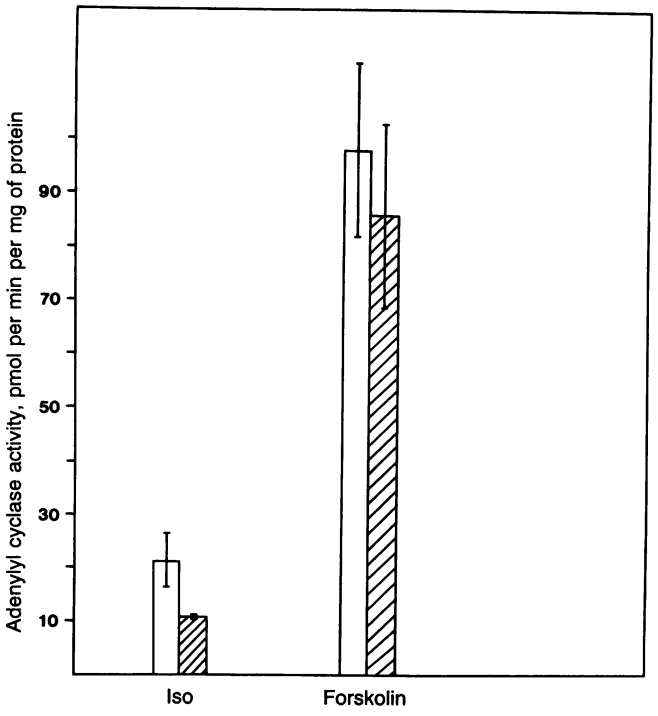

FIG. 3. Iso- and forskolin-stimulated adenylyl cyclase activity in cells expressing wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR. Membranes derived from cells expressing wild-type β_2AR (open bars) and from cells expressing [Ala^{350},Ala^{354}]β_2AR (hatched bars) were prepared and stimulated adenylyl cyclase activity was measured. Iso (0.1 mM) and forskolin (0.1 mM) were used as activators. Adenylyl cyclase activity is expressed as pmol of cAMP per min per mg of membrane protein. Data are mean ± SEM (n = 11). Unstimulated (i.e., basal) adenylyl cyclase activity of cells expressing wild-type and mutant β_2AR was 7.5 ± 1.9 and 6.0 ± 0.5 pmol of cAMP per min per mg of membrane protein, respectively.

depression (≈50%) in maximal Iso-stimulated adenylyl cyclase activity without altering agonist potency [EC_{50} for β_2AR = 55 ± 14 nM; EC_{50} for [Ala^{350},Ala^{354}]β_2AR = 54 ± 25 nM (n = 3)] (Fig. 3). The blunted adenylyl cyclase responsiveness observed with [Ala^{350},Ala^{354}]β_2AR is unlikely to be due to an alteration of the adenylyl cyclase catalytic unit in the mutant cell lines since forskolin-stimulated adenylyl cyclase activity was identical in cells expressing either wild-type or mutated receptors (Fig. 3). These findings, therefore, suggest that the mutation diminished the capacity of the β_2AR to efficiently couple with the adenylyl cyclase stimulatory pathway. This hypothesis is further supported by the observation that the proportion of β_2AR in the high-affinity state for agonist was significantly lower in cells expressing [Ala^{350},Ala^{354}]β_2AR than in cells expressing wild-type β_2AR [34 ± 7% vs. 63 ± 6% (n = 4)], suggesting that [Ala^{350}, Ala^{354}]β_2AR is somewhat uncoupled from the stimulatory guanine nucleotide-binding regulatory protein (G_s).

We have reported (21) that increased cellular cAMP levels lead to a down-regulation of β_2AR number in CHW cells and proposed that this mechanism may contribute to the down-regulation observed upon agonist stimulation of the receptor. Therefore, to determine whether the impaired coupling ability of [Ala^{350},Ala^{354}]β_2AR and the consequent reduction in agonist-stimulated cAMP production mediated by the mutant receptor might be the cause of its altered agonist-induced down-regulation, the ability of [Ala^{350},Ala^{354}]β_2AR to undergo Iso-induced down-regulation was tested in the presence of 1 mM Bt_2cAMP and 0.1 mM IBMX. Similar to the pattern observed for Iso-induced down-regulation of the mutant (Fig. 2), the down-regulation elicited in the presence of Bt_2cAMP was delayed and blunted in cells expressing [Ala^{350},Ala^{354}]β_2AR compared with wild-type β_2AR (Fig. 4). Moreover, the down-regulation induced by Bt_2cAMP alone was also significantly altered by the mutation (data not shown). Thus, a lower level of intracellular cAMP accumulation generated by [Ala^{350},Ala^{354}]β_2AR does not account for the altered pattern of down-regulation of the mutant receptor.

DISCUSSION

In the present study, we have shown that mutation of Tyr-350 and Tyr-354 of the β_2AR greatly impairs its ability to undergo agonist-induced down-regulation, thus, suggesting that these residues may be part of a molecular motif involved in the process regulating total cellular β_2AR number. Similarly, mutations of tyrosine residues located in the carboxyl tails of the low density lipoprotein (10), poly-immunoglobulin (11), and mannose-6-phosphate receptors (12) also produce significant decreases in their rates of agonist-induced internalization. The internalization of these receptors has been well characterized and involves endocytosis by way of CCVs. More recently, it has been proposed that a tyrosine residue of the mannose-6-phosphate receptor, mutation of which causes a dramatic reduction in the internalization process, may favor the interaction of the receptor with proteins of the clathrin-coated pit termed "adaptors" (22). Since the location of Tyr-350 and Tyr-354 in the carboxyl tail of the β_2AR (20 and 24 amino acids from the plasma membrane, respectively) is similar to that of the tyrosine residues involved in the internalization of the mannose-6-phosphate receptor (24 and 26 amino acids from the plasma membrane), it is tempting to speculate that Tyr-350 and Tyr-354 may contribute in a similar fashion and promote down-regulation of the receptor through a CCV-mediated pathway. As indicated above, several indirect lines of evidence support a role for clathrin-coated pits and CCV-mediated endocytosis in the regulation of cell surface β_2AR number. In this context, the involvement of tyrosine residues in the β_2AR down-regulation process may be taken as additional evidence of the participation of CCVs in the cellular trafficking of the β_2AR.

The possibility that the effects of the mutation on the β_2AR down-regulation pattern resulted from a nonspecific conformational change of the carboxyl tail of the receptor cannot be excluded. However, this possibility seems unlikely since more exhaustive mutations of adjacent regions of the carboxyl terminus did not affect the receptor down-regulation. Indeed, the truncation of the 48 most carboxyl-terminal amino acids of the receptor (from Leu-413 to Gly-365) (23) and the replacement of 13 amino acids of the β_2AR cytoplasmic tail (from Cys-327 to Leu-339) with corresponding residues from the α_2AR (R.J.L., M.B., and P. T. Campbell, unpublished observation) were found not to modify the agonist-induced down-regulation of the receptor for up to 12 hr.

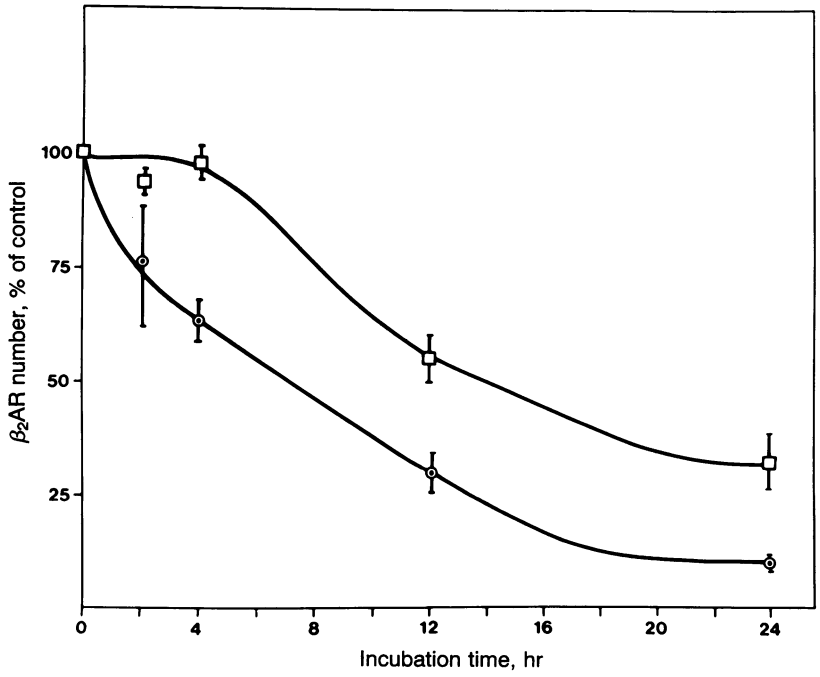

FIG. 4. Iso-induced down-regulation of β_2AR in the presence of Bt_2cAMP and IBMX in cells expressing wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR. Cells were incubated with 1 μM Iso, 1 mM Bt_2cAMP, and 0.1 mM IBMX for 0–24 hr at 37°C and whole-cell β_2AR number was determined by radioligand binding assay using [^{125}I]PIN as the radioligand. The number of receptors is expressed as percent of the receptor number present in untreated cells. Data are mean ± SEM (n = 3). ⊙, Wild-type β_2AR; □, [Ala^{350}, Ala^{354}]β_2AR.

Classically, two distinct phenomena appear to contribute to agonist-induced regulation of cell surface β_2AR number; a rapid translocation of the receptor away from the cell surface to an, as yet, ill-defined light vesicle membrane fraction (sequestration) and a longer-term loss of total cellular β_2AR (down-regulation). In several studies, down-regulation has been attributed to degradation of the receptor (2–4). However, in some cases the recovery of cell surface receptors after down-regulation has been shown to be rapid and independent of *de novo* protein synthesis (4, 24, 25). This suggests that a proportion of nondegraded receptors that are undetectable by radioligand binding assay can be recycled back to the plasma membrane in a fully functional conformation.

Mutation of Tyr-350 and Tyr-354 reduced the extent and initial rate of agonist-induced down-regulation of the β_2AR without affecting either its rate or extent of sequestration. If it is assumed that sequestration and down-regulation are sequential events, these results suggest that Tyr-350 and Tyr-354 are involved in a mechanism taking place downstream of the sequestration process. However, if a distal step in the sequential pathway was blocked by the mutation, a change in the proportion of sequestered receptors would be anticipated. As seen in Fig. 2, this was not the case and the proportion of sequestered receptor was not affected by the mutation. Therefore, it appears most likely that mutation of these tyrosine residues interferes with a down-regulation mechanism that is independent of the sequestration event. The existence of such a mechanism is supported by the observation that atypical partial agonists such as celiprolol or pindolol can induce down-regulation but not sequestration of the β_2AR (26). Moreover, it has been reported (21) that cAMP analogues induce down-regulation without sequestration of the β_2AR in CHW cells.

In addition to impairing down-regulation of the β_2AR, the mutation of Tyr-350 and Tyr-354 also affected the coupling of the receptor to G_s. This is indicated by the decreased ability of the mutated receptor to mediate stimulation of adenylyl cyclase and to form high-affinity binding with agonist (Fig. 3). However, the depressed production of cAMP mediated by [Ala^{350},Ala^{354}]β_2AR does not account for the down-regulation impairment of the mutant receptor since addition of high concentrations of Bt_2cAMP and IBMX did not restore a wild-type pattern of down-regulation (Fig. 4). The uncoupling of [Ala^{350},Ala^{354}]β_2AR appears to be the consequence of a decreased ability of the mutated receptor to functionally interact with G_S and not of a physical sequestration of the receptor away from the cell surface. Indeed the proportion of receptor expressed at the cell surface were identical for the mutated and wild-type receptors.

It, therefore, appears that similar regions of the receptor might be involved both in G_s coupling and in the process of receptor down-regulation. In this context, it is noteworthy that GTP-binding proteins have recently been proposed to play a role in the endocytotic process (27). Alternatively, the physical coupling of the receptor to G_s might directly contribute to its agonist-induced down-regulation (28–30). However, the fact that the mutation of tyrosine residues similarly located in receptors that have no known interactions with G_s also delay receptor internalization (11–13) may argue against an impairment of G_s coupling as the mechanism responsible for the delayed down-regulation process in these mutants.

In conclusion, as with membrane-bound receptors known to be endocytosed by way of CCVs, tyrosine residues located in the cytoplasmic tail of the human β_2AR appear to play an important role in the regulation of cell surface β_2AR number. These tyrosines may contribute to form a common motif of noncontiguous amino acids in various classes of transmembrane receptors that undergo a dynamic regulation of their numbers. The precise pathway involving these tyrosine residues in the β_2AR down-regulation process remains to be clarified.

We are grateful to Chantal Persechino and Kiefer Daniel for their technical assistance and to Danielle Beaudoin for the preparation of the manuscript. This work was supported in part by the Medical Research Council of Canada and the Canadian Heart Foundation. M.B. is a Scholar of the Medical Research Council of Canada. M.H. is a Fellow of the Medical Research Council of Canada.

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