New Molecular and Structural Determinants Involved in
\( \beta_2 \)-Adrenergic Receptor Desensitization and Sequestration

DELINEATION USING CHIMERIC \( \beta_2/\beta_2 \)-ADRENERGIC RECEPTORS*

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Ralf Jockers§¶, Angelo Da Silva§**, A. Donny Strosberg†, Michel Bouvier‡‡, and Stefano Marullo‡

From the CNRS-UPR 0415 and Université Paris VII, Institut Cochin de Génétique Moléculaire, 22, rue Méchain, F-75014 Paris, France and the Département de Biochimie et Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, 2900 Edouard-Montpetit, Case Postale 6128, Succursale Centre-Ville Montréal, Québec H3C 3J7, Canada.

As the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)AR) is resistant to short term agonist-promoted desensitization and sequestration, chimeric \( \beta_2/\beta_2 \) receptors were generated to identify the molecular determinants responsible for these regulatory processes in the \( \beta_2 \)AR. By exchanging single or multiple intracellular domains of the \( \beta_2 \)AR for the corresponding regions of the \( \beta_2 \)AR, we show that specific domains can be identified as additive determinants for desensitization, while sequestration is more dependent on global structural conformation. The carboxyl-terminal tail, the third and the second intracellular loops of the \( \beta_2 \)AR provided additive contributions to the desensitization observed upon short term agonist stimulation. The second intracellular loop plays a role which is as important as that of third cytoplasmic loop and carboxyl-terminal tail which had previously been identified as the major determinants of agonist-promoted desensitization. Additive contributions of the cytoplasmic domains of the \( \beta_2 \)AR were also observed for agonist-promoted sequestration. The substitution of the first and second intracellular loops and the carboxyl tail were associated with a \( \beta_2 \)-like sequestration phenotype. However, in contrast to what is observed for desensitization the co-substitution of the third cytoplasmic loop with any of the other domains completely suppressed sequestration. These results suggest that sequestration depends not only on appropriate interactions of multiple molecular determinants within the cytoplasmic region of the \( \beta_2 \)AR but also on conformational determinants that may influence their orientation.

Cellular responses to \( \beta \)-adrenergic receptor (\( \beta \)AR) stimula-

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†††Holds a fellowship from the Heart and Stroke Foundation of Canada.

§§Recient of a fellowship from the Deutsche Forschungsgemeinschaft.

**Holds a fellowship from the Heart and Stroke Foundation of Canada.

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The abbreviations used are: \( \beta \)AR, \( \beta \)-adrenergic receptor; \( \beta \)ARK, \( \beta \)-adrenergic receptor kinase; CYP, iodocyanopindolol; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CT, carboxyl tail.

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which specific domains of the β2AR have replaced their counterparts within the β2AR has already been successfully used to study their contribution to desensitization. Substitutions of the carboxyl-tail alone (11) or of both the carboxyl-tail and third cytoplasmic loop (12) of the β2AR by the corresponding region of the β3AR have been shown to confer agonist-promoted desensitization to the β2AR. However, neither of the chimeric receptors studied had desensitization profiles comparable with that of the wild-type β2AR. This suggests that other regions of the receptor are required for a complete β2AR-like desensitization.

To identify additional putative determinants involved in rapid desensitization of the β2AR and to assess the respective contribution of β2AR intracytoplasmic domains to the sequestration process, we have constructed a series of chimeric receptors in which various combinations of the β2AR intracellular loops were replaced by the corresponding domains of the β3AR.

EXPERIMENTAL PROCEDURES

Materials—[3H]ATP and [125I]CYP were from DuPont NEN and [253H]JAMP was from Amersham Corp. Isopropenine, norepinephrine, (-)-propranolol, α1-propranolol, ATP, GTP, CAMP, bovine serum albumin, phosphorpyruvate, myokinase, isobutylmethylxanthine, dibutyryl CAMP, forskolin, leupeptin, soybean trypsin inhibitor, and benzamidine were from Sigma. Pyruvate kinase was from Calbiochem. Flutamide, and streptomycin were from Life Technologies, Inc. DMEM, fetal bovine serum, PBS, trypsin-EDTA, Genetecin (G418), penicillin, and streptomycin were from Life Technologies, Inc. CGP12177A and bupranolol were generous gifts from Ciba Geigy and Schwarz Pharma, respectively. BRL37344 was a gift from SmithKline Beecham Pharmaceutical, and ICI118551 and ICI201651 were gifts from Imperial Chemical Industries Zeneca.

Construction of Chimeric Receptors and Cell Culture—Chimeric β2/β3AR coding regions illustrated in Fig. 1 were constructed using the following approaches: (i) for the exchange of the third cytoplasmic loop and the carboxyl terminus, silent restriction sites were generated by the Bio-Rad protein assay system with bovine serum albumin as standard. Sequestration Assays—Cells grown in 75-cm² flasks were incubated in the presence of 10 μM isoproterenol in DMEM containing 10 μM ascorbic acid or the vehicle alone for the indicated periods of time. The flasks were then placed on ice, washed twice with ice-cold PBS, and the cells detached mechanically in a buffer containing 5 mM Tris, 2 mM EDTA, pH 7.4, 5 mM MgCl₂, 50 μg/ml leupeptin, and 10 μg/ml benzamidine (buffer A). Cell suspensions were homogenized with a Polytron homogenizer (Julke & Undel Ultra-Turrax T25) for 5 s at maximal setting. The lysate was centrifuged at 450 × g for 5 min at 4 °C. The supernatant was layered on top of a 35% sucrose cushion and centrifuged at 150,000 × g for 90 min. As reported previously (15), the light membrane vesicular fraction was found at the 0–35% interface, whereas the plasma membrane fraction sedimented at the bottom of the sucrose cushion. Each fraction was collected, diluted in buffer A and centrifuged at 200,000 × g for 60 min. The pellet membranes were resuspended in 50 mM Tris, 5 mM MgCl₂, pH 7.4, and used immediately for radioligand binding assays. Binding assays were carried out as described above but using membrane preparations instead of cell suspensions. Sequestration assays for the wild type β2AR using this technique were identical to those measured in whole cell binding assays in which sequestration is defined by the number of [253H] JAMP binding sites inaccessible to the hydrophilic ligand CGP12177A (data not shown).

Dadenyl Cyclase Assays—Cells grown in 75-cm² flasks were incubated in DMEM containing 10 μM ascorbic acid with or without 10 μM isoproterenol for the indicated periods of time. Incubations were stopped by washing the cells twice with ice-cold PBS. Cells were then detached mechanically and homogenized in ice-cold buffer A using a polytron homogenizer (Julke & Undel Ultra-Turrax T25). Lysates were centrifuged at 450 × g for 5 min at 4 °C. Supernatants were centrifuged at 43,000 × g for 20 min at 4 °C and the pellets washed twice in buffer A. The washed membranes were then resuspended in a buffer containing 75 mM Tris, pH 7.4, 5 mM MgCl₂, 2 mM EDTA, and protease inhibitors.

Adenylyl cyclase activity was measured on these membrane preparations according to the method of Salomon et al. (16). Briefly, the reaction mixture contained: 20 μl of membrane preparation (2–6 μg of protein), 45 mM Tris (pH 7.4), 1.2 mM ATP, 0.12 mM GMP, 0.003 mM GTP, 0.1 mM CAMP, 0.1 mM isobutylmethylxanthine, 1 μCi of [32P] ATP, 2.8 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase and 1 unit of myokinase in a final volume of 50 μl. Enzymatic activity was determined in the presence of 0–1 mM isoproterenol for 30 min at 37 °C. The reactions were terminated by the addition of 1 ml of ice-cold stop solution containing 0.4 M Tris, 0.3 mM GMP, and 25,000 cpm [3H]JAMP. The cAMP was then isolated by sequential chromatography on Dowex cation exchange resin and aluminum oxide. Data are expressed as picomoles of cAMP produced per min per mg of protein. The results of three to eight experiments were fitted simultaneously (using the nonlinear least squares regression program SigmaPlot) so as to give an averaged best fit value.

Determination of Intracellular Cyclic AMP Levels—Cells were washed once in PBS and incubated in the absence or presence of 10 μM isoproterenol or CGP12177A for 15 min at 37 °C in PBS 0.5 mM isobutylmethylxanthine, 0.5 mM ascorbic acid. The incubation buffer was discarded and cells lysed in 1 ml NaOH for 30 min at 37 °C. The lystate was neutralized with 1 M acetic acid and centrifuged in a microfuge at maximum speed for 5 min. The supernatant was used for cAMP determination using [3H]JAMP radiommunoassay system (Amersham Corp.).

RESULTS

Pharmacological Characterization of the β2/β3AR Chimeras—In order to assess the contribution of the various cytoplasmic domains of the β2AR to agonist-promoted uncoupling and sequestration, chimeric receptors were constructed which comprised extracellular and transmembrane regions of β3AR origin and various combinations of intracellular domains from β2AR and β3AR. Conserved amino acid sequences between the β2/β3AR Chimeras—In order to assess the contribution of the various cytoplasmic domains of the β2AR to agonist-promoted uncoupling and sequestration, chimeric receptors were constructed which comprised extracellular and transmembrane regions of β3AR origin and various combinations of intracellular domains from β2AR and β3AR. Conserved amino acid sequences between the β2/β3AR Chimeras—In order to assess the contribution of the various cytoplasmic domains of the β2AR to agonist-promoted uncoupling and sequestration, chimeric receptors were constructed which comprised extracellular and transmembrane regions of β3AR origin and various combinations of intracellular domains from β2AR and β3AR. Conserved amino acid sequences between the β2/β3AR Chimeras—In order to assess the contribution of the various cytoplasmic domains of the β2AR to agonist-promoted uncoupling and sequestration, chimeric receptors were constructed which comprised extracellular and transmembrane regions of β3AR origin and various combinations of intracellular domains from β2AR and β3AR. Conserved amino acid sequences between the β2/β3AR Chimeras—In order to assess the contribution of the various cytoplasmic domains of the β2AR to agonist-promoted uncoupling and sequestration, chimeric receptors were constructed which comprised extracellular and transmembrane regions of β3AR origin and various combinations of intracellular domains from β2AR and β3AR. Conserved amino acid sequences between...
and the $\beta_2$AR within the putative transmembrane regions and the corresponding junctions with intracellular loops facilitated the exchange of intracellular domains (Fig. 1). For the chimeric receptor identified as $\beta_3$-2222, all the cytoplasmic domains of the $\beta_2$AR were substituted by those of the $\beta_3$AR. Eight additional $\beta_3$/$\beta_2$AR chimeric receptors were generated. Clones isolated from Ltk$^-$ cells stably expressing between 150 and 960 fmol of receptor/mg of protein were selected for further study. As shown in Table I, all the chimeras constructed retained affinity constants for $[125I]$CYP comparable with that of the wild type $\beta_3$AR (500–3000 pM), which is significantly higher than that of the $\beta_2$AR (51 pM). Further characterization of $\beta_3$-2222 clearly showed that, despite a moderate decrease in the affinity for several ligands, this receptor which contains the largest contribution of $\beta_2$AR sequences, still retained a general order of potency for $\beta_2$-adrenergic ligands which is characteristic of the $\beta_3$AR (Table II). Particularly revealing is the relatively low affinity of the $\beta_3$-2222 for isoproterenol, alpranolol, bupranolol, and ICI118551. Furthermore, CGP12177A which is an antagonist at the $\beta_2$AR but has partial agonistic properties toward the $\beta_3$AR, stimulated adenylyl cyclase with an intrinsic activity of 0.6 in cells expressing $\beta_3$-2222, thus confirming the $\beta_3$AR-like pharmacology of $\beta_3$-2222 (Table II).

**Fig. 1.** A, topological model of the human $\beta_3$AR. Conserved amino acid residues between the $\beta_2$AR and $\beta_3$AR sequences are represented by filled circles. Arrows indicate the connecting sites between the $\beta_2$AR core sequence and the substituted $\beta_3$AR cytoplasmic domains in the chimeric receptors. B, sequence comparison of the cytoplasmic domains of $\beta_2$AR and $\beta_3$AR. Hyphens indicate identity with the $\beta_3$AR sequence. Triangles indicate protein kinase A phosphorylation sites. Stars indicate potential phosphorylation sites for ARK. Motifs identified as potential determinants of sequestration are overlined.
This confirms that the β2AR is largely resistant to rapid agonist-induced desensitization compared with the β1AR.

Substitution of the first cytoplasmic loop (i1) of the β1AR with the corresponding region from β2AR did not confer any desensitization phenotype as prestimulation of cells expressing this mutant receptor, for 2 or 15 min, did not affect either the dose-response curves or the maximal stimulation (Fig. 2, panel B). As expected from previous studies, substitution of either i3 (Fig. 2, panel C) or CT (Fig. 2, panel D) conferred desensitization profiles that are characterized mainly by rightward shifts of the dose-response curves that became clearly evident following 15 min of prestimulation. Moreover, as can be seen in panel F, the contribution of these two domains to the desensitization pattern appear to be additive. Indeed, the desensitization of the β2AR was faster and larger than that conferred by single substitution of CT alone (compare panels G and D). Interestingly, the reduction in agonist-stimulated adenylyl cyclase activity in cells expressing β2AR was even faster than that observed in cells expressing β2AR. Indeed, a 25% reduction in the maximal stimulation was already evident following a 2-min preincubation period for the β2AR, whereas no change in the maximal stimulation was observed at this time for the β3AR. An additive effect of i2 on desensitization is also evident when comparing β2AR with β3AR (compare panels E and H).

The apparently additive contribution of the β2AR i2, i3, and CT to the desensitization profiles of the chimeric receptors can be easily appreciated by looking at Fig. 2. Indeed, it can be seen that co-substitution of these three domains leads to a progressive increase in the overall extent of desensitization. However, a quantitative assessment of the additivity is rendered difficult by the fact that desensitization is reflected by changes in two parameters, i.e. a reduction in the maximal stimulation and a rightward shift of the dose-response curves. The assessment of the dose-response shifts is further complicated by the fact that not all chimeras have the same efficacy to stimulate the adenylyl cyclase activity under basal conditions and that in several cases, the desensitized adenylyl cyclase activity did not reach a plateau at the highest isoproterenol concentration used (1 mM), thus making mathematical analysis more difficult. Therefore, the reduction in adenylyl cyclase activity measured for a stim-
ulating concentration of isoproterenol equal to its EC50 for a given chimera was used as an index of the dose-response rightward shift. Fig. 3 illustrates the amplitude of the changes in stimulation at maximal concentration and at the EC50 for all the chimeric receptors following a desensitization of 15 min. The effects of the single substitutions of i2, i3, or CT on the desensitization are reflected mainly by a reduction of the response at the EC50 with only marginal effects on the maximal stimulation. Double or triple substitution of these domains conferred agonist-dependent reduction of both the maximal stimulation and of the stimulation at the EC50. Interestingly, the substitution of i1, which has no effect on the desensitization pattern by itself, conferred a slight negative effect on the desensitization of the maximal response when co-substituted along with other cytoplasmic domain of β2 origin (compare β3-3232 and β3-3222 with β3-2232 and β3-2222). This might result from an unfavorable conformational effect, since the affinity for [125I]CYP was also significantly reduced by the co-substitution of i1 (Table I). Since β2-2222 and β2-2232 also have slightly reduced affinity for isoproterenol, as indicated by their higher Kact when compared with β2AR (Table I), it might be suggested that the lower extent of the maximal response desensitization of these chimeras is an underestimation resulting from the potentially nonsaturating conditions used. However, this is highly unlikely, since β3-3232 and β3-3222 have similar elevated Kact, and yet they display the greatest desensitization of the maximal response approaching the level observed for the β2AR.

Molecular Determinants of Isoproterenol-dependent Sequestration—As reported previously (17), agonist stimulation leads to a rapid and time-dependent translocation of β2AR from the plasma membrane to a light vesicular fraction (Fig. 4). In contrast, no such sequestration is observed in cells expressing the β3AR. In fact, isoproterenol promoted an apparent enrichment of the plasma membrane fraction in β3-binding sites (expressed in Fig. 4 as negative sequestration). In previous attempts aimed at identifying molecular determinants of sequestration, the exchange of intracellular domains of the β3AR with the corresponding regions of the β2AR (11, 12) led to apparently contradictory observations: the exchange of the CT alone partially restored agonist-promoted sequestration, but no sequestration could be observed in a chimeric receptor harboring both the i3 and the CT of the β2AR. Therefore, we tested whether any single or multiple exchanges between intracellular regions of receptors could restore a sequestration profile similar to that observed for the β2AR.

Sequestration of the β2AR in Ltk- cells reached a maximum of 34% after 15 min of isoproterenol stimulation. Thus, we first screened L cells expressing the chimeric receptors described above by measuring sequestration following incubation with 10 μM isoproterenol for 5 and 15 min (Fig. 4). The single substitution of either CT, i2 or i1 of the β2AR with the corresponding regions of the β2AR partially restored agonist-promoted sequestration (β2-3332: 12%; β2-3233: 8%; β2-2333: 7%, following a stimulation of 15 min). In contrast, substitution of i3 had no apparent effect on the sequestration pattern. Positive effects on
the concentration of isoproterenol used to promote sequestration to up to 1 mM did not induce any sequestration of \( \beta_2 \)-2222 (data not shown).

To further characterize chimeric receptors showing positive sequestration, this process was studied for longer periods of time. For the three single substitutions (\( \beta_2 \)-2333, \( \beta_2 \)-3232, \( \beta_2 \)-3332), agonist-promoted sequestration reached its maximum between 15 and 30 min of stimulation (10%-15%) and remained at that level for up to 60 min (Fig. 5). Although sequestration was observed for these three chimeras, the level of sequestration never reached that observed for the \( \beta_2 \)-AR (30%). In contrast, sequestration of \( \beta_2 \)-2332 and \( \beta_2 \)-2232 attained levels observed for the \( \beta_2 \)-AR albeit with somewhat slower kinetics. Indeed, sequestration levels were equivalent to those of the \( \beta_2 \)-AR after 60 min of stimulation. These results suggest that three \( \beta_2 \)-AR cytoplasmic domains, 1I, 12, and CT, provide positive sequestration signals that may be somewhat additive but are not sufficient to restore an entirely normal \( \beta_2 \)-AR sequestration profile.

**DISCUSSION**

Despite extensive investigation during the past years, molecular mechanisms involved in short term \( \beta_2 \)-AR regulation have not been completely elucidated. We took advantage of the high degree of homology existing between the \( \beta_2 \)-AR and the \( \beta_3 \)-AR (69% within putative membrane spanning domains and corresponding junctions with intracellular loops) and of their distinct profile of regulation to identify novel molecular determinants of receptor desensitization and sequestration. Current hypothetical models suggest that molecular determinants of \( \beta_2 \)-AR regulation are located in intracellular domains (18). Sequence homology between \( \beta_2 \)-AR and \( \beta_3 \)-AR facilitated the exchange of unmodified intracellular domains and the construction of functional chimeric receptors. We previously showed that the chimeric receptor strategy is particularly adapted to study molecular basis of receptor function (19). This approach, complementary to site-directed mutagenesis studies, allows assessment of the contribution of entire structural domains without preconceived notions of the precise residues involved.

The chimeric receptors constructed in the present study conserve pharmacological properties characteristic of the \( \beta_2 \)-AR. In particular, \( \beta_2 \)-2222 which contains the largest proportion of \( \beta_2 \)-AR derived sequence maintained all the pharmacological trademarks of the \( \beta_2 \)-AR including the agonistic properties of the \( \beta_3 \)-AR antagonist CGP12177A. Previous studies, based on molecular modelling and pharmacological characterization of the \( \beta_2 \)- and \( \beta_3 \)-AR suggested that \( \beta_2 \)-antagonists with \( \beta_3 \)-agonist properties, such as CGP12177A, may adopt a stacked conformation in the \( \beta_2 \)-AR binding pocket, leading to antagonistic effects while they would adopt an extended conformation in the less encumbered \( \beta_3 \)-binding site. This last conformation may allow interactions with specific residues implicated in signal transduction (20). The \( \beta_2 \)-like pharmacological properties maintained in \( \beta_2 \)-2222 suggest that the intracellular domains do not affect the overall organization of the binding pocket determined by the positioning and the orientation of the transmembrane domains.

As reported previously (12), substitution of the cytoplasmic domains of the \( \beta_2 \)-AR with those of \( \beta_3 \)-AR containing all known specific consensus sequences for receptor phosphorylation by \( \beta \)-ARK and protein kinase A (I3, CT) (4, 5, 15, 21, 22) failed to confer a \( \beta_2 \)-AR-like desensitization profile. The present report clearly shows that additional molecular determinants involved in receptor desensitization are also present in the \( \beta_2 \)-AR I2. The presence of this domain alone is sufficient to confer a desensitization level at least equivalent to that provided by CT and I3. Furthermore, when substituted in combination with the other
domains, additive effects on the level of agonist-promoted desensitization were found. Consistent with the contribution of i2 to receptor desensitization is the observation that Phe-139 located in i2 of the β2AR is apparently involved in G-protein coupling (8). The recent finding that phosphorylation of Tyr-141 within the β2AR i2 favors its coupling with Gs (23) also suggests that this domain plays an important role in the regulation of receptor-Gs interaction. The contribution of i2 to the desensitization process could result from its interaction with previously identified proteins, which regulate receptor function, such as βARK or β-arrestin thus stabilizing their interactions with domains already characterized. Alternatively, i2 may contain new unidentified sites which promote receptor uncoupling. The observation that substitution of i2 alone is sufficient to confer receptor desensitization would support the latter.

All molecular determinants of β2AR uncoupling identified so far correspond to phosphorylation targets for protein kinases.

In a previous report mutation of all putative βARK, protein kinase A and protein kinase C phosphorylation sites significantly reduced agonist-promoted phosphorylation and desensitization but did not completely abolish them (5). This is consistent with the idea that additional phosphorylation sites may exist and be involved in receptor desensitization. Two serine residues Ser-137 and Ser-143 present in i2 of β2AR are absent from the β3AR. These residues might be the target of another kinase. One serine (Ser-137) is contained in the potential phosphorylation consensus site S/T P X K/R, which has been shown to be a preferred substrate for cdc2 kinase (24). Additional experiments are required to assess whether this region contains phosphorylation sites involved in receptor uncoupling and to identify the putative kinase participating in such regulation.

Previous studies have suggested the existence of several motifs located in various cytoplasmic domains of the β2AR involved in sequestration. However, no clear connection could be established between these motifs that leads to an unequivocal identification of the molecular determinants triggering the sequestration process. In their studies, Hausdorff et al. (25) showed that site-directed mutagenesis of a subset of serine residues, believed to be βARK phosphorylation sites, blocked agonist-promoted sequestration. In particular, substitution of Ser-356 and Ser-364 by glycine residues completely blocked sequestration. However, mutations of additional serines and threonines in this region restored a normal sequestration phenotype (4). The authors concluded that Ser-356 and Ser-364 are not required for sequestration but that their mutation leads to conformational changes interfering with the sequestration process. Also suggesting that receptor conformation may influence sequestration is the recent report by Green and Liggett (9), indicating that a proline-rich sequence located in the third cytoplasmic loop of the β2AR prevents the efficient sequestration of this receptor subtype. Recently, a tyrosine residue (Tyr-326) located at the interface between the seventh transmembrane domain and the carboxyl tail has been proposed as a specific determinant for β2AR sequestration (10). Although this residue may be required, it is certainly not sufficient to confer an agonist-promoted sequestration phenotype. Indeed, a tyro-
sine residue within a NPXXY motif identical to that of the \( \beta_2 \text{AR} \) is also present in a similar position in the \( \beta_3 \text{AR} \). However, the \( \beta_2 \text{AR} \) subtype is not sequestered upon agonist stimulation (11, 12). In addition, mutation of the tyrosine residue located in the NPXXY motif of the gastrin-releasing peptide receptor or of the Type 1 angiotensin II receptor did not affect their agonist-promoted sequestration arguing against a general role for this sequence (26, 27). More recently, Ferguson et al. (28) proposed that the reduction of sequestration caused by the mutation of Tyr-326 in the \( \beta_2 \text{AR} \) resulted from the inability of this mutant receptor to act as a substrate for \( \beta \text{ARK} \). They proposed that \( \beta \text{ARK} \)-mediated phosphorylation facilitates \( \beta_2 \text{AR} \) sequestration. Although that may be the case, it is clear from previous studies that phosphorylation by \( \beta \text{ARK} \) is not an absolute requirement nor is it the signal initiating the sequestration process. Indeed, it has been shown that \( \beta_2 \text{AR} \) lacking all putative \( \beta \text{ARK} \) phosphorylation sites can readily be sequestered upon agonist stimulation (3, 7, 10, 28). The presence of an hydrophobic residue in the DRYXX(V)XXP2 sequence (where Z is the hydrophobic residue) within the second cytoplasmic loop of the \( \beta_2 \text{AR} \) has also been proposed as being important for receptor sequestration (8). Such a hydrophobic residue is conserved in identical position in the \( \beta_2 \text{AR} \) (DRYLVATNPL), suggesting that the presence of this residue is not sufficient to facilitate agonist-promoted sequestration.

Our data support the notion that interaction between multiple intracellular domains of the \( \beta_2 \text{AR} \) contribute to sequestration phenotypes. Clearly, none of the cytoplasmic domains (which contain the various sequestration signals previously proposed), when substituted alone, could confer a \( \beta_2 \text{AR} \)-like sequestration pattern. In fact, i1, i2 and CT allowed very moderate agonist-promoted sequestration, while the association of the second intracytoplasmic loop with the carboxyl terminus of the \( \beta_2 \text{AR} \) in the chimeric \( 2\text{AR} \) resulted from the inability of a specific domain resulting from specific interactions among intra-cytoplasmic domains is required for proper sequestration. In conclusion, we have shown that in addition to the carboxyl tail and the third cytoplasmic loop, the second cytoplasmic loop of the \( \beta_2 \text{AR} \) is involved in the process of agonist-promoted desensitization. Also, sequestration does not merely depend on the presence of specific domains (i.e. i2 and CT) but largely relies on the proper arrangement of all the cytoplasmic domains.

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