Dual Role of the $\beta_2$-Adrenergic Receptor C Terminus for the Binding of $\beta$-Arrestin and Receptor Internalization*

Received for publication, August 6, 2008, and in revised form, September 16, 2008. Published, JBC Papers in Press, September 18, 2008, DOI 10.1074/jbc.M806086200

Cornelius Krasel1,2, Ulrike Zabel1, Kristina Lorenz3, Susanne Reiner1, Suleiman Al-Sabah1, and Martin J. Lohse3

From the 1Institute of Pharmacology and Toxicology, Versbacher Strasse 9, D-97078 Wuerzburg, Germany and the 3School of Pharmacy, University of Reading, Whiteknights, Reading RG6 6AJ, United Kingdom

Homologous desensitization of $\beta_2$-adrenergic and other G-protein-coupled receptors is a two-step process. After phosphorylation of agonist-occupied receptors by G-protein-coupled receptor kinases, they bind $\beta$-arrestins, which triggers desensitization and internalization of the receptors. Because it is not known which regions of the receptor are recognized by $\beta$-arrestins, we have investigated $\beta$-arrestin interaction and internalization of a set of mutants of the human $\beta_2$-adrenergic receptor. Mutation of the four serine/threonine residues distal to residue 381 to the plasma membrane, and receptor internalization. Mutation of all seven serine/threonine residues distal to residue 381 did not affect agonist-induced receptor-$\beta$-arrestin2 interaction as revealed by fluorescence resonance energy transfer (FRET), translocation of $\beta$-arrestin2 to the plasma membrane, and receptor internalization. Mutation of all seven serine/threonine residues distal to residue 381 did not affect the loss of agonist-induced receptor-$\beta$-arrestin2 interaction as revealed by fluorescence resonance energy transfer (FRET), translocation of $\beta$-arrestin2 to the plasma membrane, and receptor internalization. A $\beta_2$-adrenergic receptor truncated distal to residue 381 interacted normally with $\beta$-arrestin2, whereas its ability to internalize in an agonist-dependent manner was compromised. A similar impairment of internalization was observed when only the last eight residues of the C terminus were deleted. Our experiments show that the C terminus distal to residue 381 does not affect the initial interaction between receptor and $\beta$-arrestin, but its last eight amino acids facilitate receptor internalization in concert with $\beta$-arrestin2.

The interaction of $\beta$-arrestins with G-protein-coupled receptors is a prerequisite for at least three different processes: homologous desensitization (1), activation of tyrosine kinase-mediated signaling pathways (2), and receptor internalization (3). This interaction requires the phosphorylation of the receptor by G-protein-coupled receptor kinases (GRKs)3 and (at least for some receptors) the continuous presence of agonist (4). GRKs are unique among serine/threonine kinases in that they do not recognize a well-defined consensus sequence but instead show high specificity for agonist-activated receptors. This lack of a consensus sequence has made mapping of phosphorylated residues in G-protein-coupled receptors difficult. For example, the residues phosphorylated by GRK2 in the $\beta_2$-adrenergic receptor have been mapped to the C terminus, either between amino acids 384 and 411 (5) or between amino acids 355 and 364 (6–10). It is now clear from a variety of studies (see Ref. 11 for a review) that $\beta$-arrestins do not simply act as “phosphoreceptor-specific antibodies.” Rather, the interaction of $\beta$-arrestins with GRK-phosphorylated receptors is believed to lead to a conformational change in the $\beta$-arrestin molecule, which enables it to bind to other parts of the receptor with higher affinity. Furthermore, it has also been demonstrated that $\beta$-arrestins sense the activated conformation of the receptor (4, 12). For example, $\beta$-arrestin mutants have been described that do not require GRK-mediated phosphorylation to bind to a receptor but still only interact with a receptor when it is activated by agonist (12). The molecular determinants on the receptor molecule for this exquisite selectivity are still unknown. Pull-down assays using fusion proteins have suggested a role for the third intracellular loop for arrestin interaction with muscarinic, $\alpha_2$-adrenergic, $\delta$-opioid, serotonin2A, and vasopressin V1 receptors, respectively (13–19). Arrestin translocation assays have shown a role for the second intracellular loop of the 5-HT2C, $\beta_2$-adrenergic, $\alpha_2$-adrenergic, and neuropeptide Y2 receptors (20). Moreover, it has been reported that the C terminus of the dopamine D1 receptor imposed an inhibitory effect on arrestin binding to the receptor, which could be relieved by its GRK-mediated phosphorylation (21).

In this study, we set out to investigate the role of the $\beta_2$-adrenergic receptor ($\beta_2$AR) C terminus for the interaction with $\beta$-arrestin2. To this end, we assessed the internalization and ability to interact with $\beta$-arrestins of a set of $\beta_2$AR mutants containing various truncation or substitution mutations in the C-terminal tail.

EXPERIMENTAL PROCEDURES

DNA Constructs and Transient Expression in HEK293 Cells—The amino acid sequences of the various $\beta_2$AR mutants are shown in Fig. 1. The phosphorylation-deficient $\beta_2$AR (PD), lacking all putative phosphorylation sites for GRKs, but not

---

* This work was supported in part by SFB487 "Regulatory Membrane Proteins," by a Leibniz grant from the DFG, and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by New Investigator Grant BB/D012902/1 from the Biotechnology and Biological Sciences Research Council.

2 To whom correspondence should be addressed: School of Pharmacy, Whiteknights, P. O. Box 228, Reading RG6 6AJ, United Kingdom. E-mail: c.krasel@reading.ac.uk.

3 The abbreviations used are: GRK, G-protein-coupled receptor kinase; $\beta_2$AR, $\beta_2$-adrenergic receptor; $\beta_2$AR-YFP, $\beta_2$-adrenergic receptor tagged with yellow fluorescent protein at the C terminus; $\beta$Arr2, $\beta$-arrestin2; $\beta$Arr2-CFP, $\beta$-arrestin2 C-terminally tagged with cyan fluorescent protein; $\beta$Arr2-YFP, $\beta$-arrestin2 C-terminally tagged with yellow fluorescent protein; FRET, fluorescence resonance energy transfer; HEK, human embryonic kidney; 125I-CYP, 125I-cyanopindolol; PD, phosphorylation-deficient; ANOVA, analysis of variance; WT, wild type.
**RESULTS**

**Ligand Binding of the Receptor Mutants**—It has previously been shown that a mutant β2AR, in which all eleven serine and threonine residues distal to amino acid 354 are mutated, is not phosphorylated by GRK in response to agonist treatment (22).

Starting from this phosphorylation-deficient (PD) mutant, we constructed further mutants (sequences are shown in Fig. 1) to analyze the role of the C terminus for receptor internalization in more detail. All receptor mutants were either N-terminally tagged with a FLAG tag, or C-terminally tagged with YFP as described for the wild-type receptor (4). All DNAs were cloned in the pcDNA3 expression vector (Invitrogen, Karlsruhe, Germany) and verified by sequencing. pcDNA3 expression vectors for CFP- and YFP-tagged bovine β-arrestin2. Blotting for GFP (Clontech, Saint-Germain-en-Laye, France) showed that GRK2 was overexpressed by at least a factor of 10. The amount of overexpression of arrestin, as receptor expression levels could affect the extent of internalization.

**Receptor Internalization Assays**—HEK293 cells were transiently transfected with FLAG-tagged wild-type or mutant β2AR and human GRK2 were labeled with [32P]orthophosphate, treated (or not) with 1 μM isoproterenol for 5 min, and receptors were immunoprecipitated with M2 anti-FLAG-Sepharose (Sigma-Aldrich) and subjected to SDS-polyacrylamide gel electrophoresis, as described previously (24, 25). Incorporated radioactivity was quantified by phosphorimager analysis. Equal expression of the mutant receptors was verified in parallel by Western blots with M2-anti-FLAG antibody (Sigma-Aldrich).

**Adenylyl Cyclase Assays**—To determine receptor-stimulated adenyl cyclase activity, cAMP production by freshly prepared membranes (100 μg of protein) from transiently transfected HEK293 cells was measured for 30 min as described (23). Care was taken that similar expression levels were achieved for wild-type and mutant receptors. Concentration-response curves (3 nM to 50 μM isoproterenol) were analyzed by nonlinear curve fitting.

**Measurement of Receptor-Arrestin Interaction—**FRET was measured in single HEK293 cells as described previously (4). Briefly, cells were transiently cotransfected with YFP-tagged wild-type or mutant β2AR, βArr2-CFP, and GRK2. Cells were observed on an Axiovert200 inverted microscope and fluorescence intensities at 480 ± 20 and 335 ± 15 nm were measured with a dual emission photometric system (Till Photonics, Gräfelfing, Germany). Kinetics was fitted using the monoexponential function built into Origin (OriginLab, Northampton, MA).

**β-Arrestin translocation was analyzed in HEK293 cells,** which were cotransfected with FLAG-tagged wild-type or mutant β2AR, βArr2-CFP, and GRK2. The subcellular distribution of βArr2-YFP was monitored by live cell confocal microscopy performed on a Leica TCS SP2 system as described (4).

**Radioligand Binding Assays**—Binding of [125I]-cyanoopindolol ([125I]-CYP; GE Healthcare, Munich, Germany) to FLAG-tagged wild-type or mutant β2AR was determined in membranes prepared from transiently transfected HEK293 cells as described (23). K_d values for [125I]-CYP binding in saturation experiments (5–400 pm) were calculated by nonlinear curve fitting. K_d values for isoproterenol were determined in competition binding experiments using 10^{-11}-10^{-8} m isoproterenol. 10 μM GTP was included to convert all receptors into a low affinity state.

**FIGURE 1. Sequence alignment of the human β2AR mutants used in this study.** Shown are all residues starting at phenylalanine 336, which is located at the beginning of the intracellular C-terminal tail. Serine and threonine residues are marked with arrowheads above the wild-type sequence, and amino acids used for replacement are shaded in the corresponding mutant sequences.

- **PD:** 336_345
- **PD-N:** 336_345
- **PD-C:** 336_345
- **ΔC2:** 336_345
- **ΔC2-PD:** 336_345
- **ΔAsn405:** 336_345
somewhat (Table 1). Similar findings have been reported previously for the PD (22) and the PD-N (6) mutant.

**β-Arrestin Interaction and Internalization of Full-length Receptors**—We and others have previously shown that a mutant β2-adrenergic receptor, which lacks all serine and threonine residues in the C terminus (22), does not interact with β-arrestin2 (4). This interaction is frequently assessed by confocal microscopy in which agonist-induced translocation of β-arrestin2-GFP fusion proteins to the plasma membrane is visualized. In HEK293 cells transiently transfected with β2AR and βArr2-YFP, stimulation of the cells with 10 μM isoproterenol led to a pronounced translocation of fluorescence after 3 min of isoproterenol treatment (Fig. 2). This translocation was not apparent when the PD mutant was used instead of the wild-type receptor (Fig. 2). Similarly, the PD-N mutant, in which only the proximal group of four serine/threonine residues (between residues 355 and 364) was mutated, failed to recruit βArr2-YFP to the plasma membrane (Fig. 2). In contrast, the PD-C mutant, in which all serines/threonines of the distal cluster (residues 384 and following) were mutated, behaved very much like wild-type receptors with respect to βArr2-YFP translocation (Fig. 2).

In a second approach, we analyzed the receptor:β-arrestin interaction in transiently transfected HEK293 cells by fluorescence resonance energy transfer between YFP-tagged receptor and CFP-tagged β-arrestin2 (4). In this assay, the receptor:β-arrestin interaction is again strictly dependent on GRK phosphorylation of the receptor (4). Fig. 3A shows a representative time course of interaction between wild-type β2AR-YFP and βArr2-CFP in the presence of GRK2, which occurred with a t1/2 of 8.3 s (Table 2). No agonist-induced FRET between the YFP-tagged β2AR-PD and βArr2-CFP was observed (Fig. 3B). Similarly, the YFP-tagged PD-N receptor showed only very little interaction with βArr2-CFP, which was barely detectable in the FRET assay (Fig. 3C). In contrast, the YFP-tagged PD-C mutant produced a clear FRET signal with βArr2-CFP, which was barely detectable in the FRET assay (Fig. 3C). Notably, the FRET amplitude produced a clear FRET signal with βArr2-CFP, which was barely detectable in the FRET assay (Fig. 3C). Notably, the FRET amplitude obtained with the PD-C construct was reproducibly smaller than with the wild-type receptor, similar to the differences that we observed previously between the two agonists isoproterenol and norepinephrine. This suggests that the C terminus of the PD-C receptor assumes a different conformation compared with the WT when binding β-arrestin2.

When the receptor was repeatedly stimulated, β-arrestin2 bound fast and with similar kinetics to both WT and PD-C receptors (Table 2, t1/2 of 1.1 s for the WT receptor and 1.5 s for the PD-C receptor). We have shown earlier (4) that such rapid β-arrestin2 binding is most likely due to binding to already phosphorylated receptors. Notably, the FRET amplitude obtained with the PD-C construct was reproducibly smaller than with the wild-type receptor, similar to the differences that we observed previously between the two agonists isoproterenol and norepinephrine. This suggests that the C terminus of the PD-C receptor assumes a different conformation compared with the WT when binding β-arrestin2.

Next, we analyzed agonist-stimulated internalization of the β2-adrenergic receptor, which is a β-arrestin-dependent process and should, therefore, be affected in the PD and PD-N receptor mutants that did not interact with β-arrestin2 in our previous experiments. In fact, the internalization behavior of these mutants corresponded well with their ability to interact with β-arrestin: while the PD-C mutant internalized with a time course similar to the wild-type receptor, internalization of the PD and PD-N mutants was virtually abolished (Fig. 4A). A similar internalization pattern for the PD mutant has been reported previously (26), in contrast to the original investigation with this mutant (22). Taken together, our data support the proximal serine/threonine cluster (between residues 355 and 364) being the critical site for GRK phosphorylation and subsequent efficient β-arrestin2 binding.

Interestingly, internalization of the PD and PD-N mutant receptors could be partially rescued by overexpression of

---

**TABLE 1**

| Ligand binding parameters in membranes from HEK293 cells transiently expressing FLAG- or YFP-tagged wild-type or mutant human β2AR |
|--------------------------|-----------------|-----------------|-----------------|
| Binding constants for 125I-CYP and isoproterenol were determined from saturation experiments and competition experiments, respectively. Each value represents the mean ± S.E. of at least six (125I-CYP binding) or four (isoproterenol competition) experiments performed in triplicate. |

| Receptor | Kd (125I-CYP binding) | Kd (isoproterenol competition) |
|----------|------------------------|--------------------------------|
| WT       | 10.48 ± 0.14           | 6.44 ± 0.10                   |
| PD       | 10.69 ± 0.13           | 6.73 ± 0.11                   |
| PD-N     | 10.51 ± 0.10           | 6.50 ± 0.11                   |
| PD-C     | 10.67 ± 0.14           | 6.69 ± 0.14                   |
| ΔC2      | 10.58 ± 0.09           | 6.52 ± 0.13                   |
| ΔC2-PD   | 10.62 ± 0.13           | 6.52 ± 0.18                   |
| ΔAsn405  | 10.17 ± 0.12           | 6.01 ± 0.08                  |
| WT-YFP   | 10.28 ± 0.09           | 6.05 ± 0.07                  |
| ΔC2-YFP  | 10.04 ± 0.11           | 6.03 ± 0.07                  |

* Significant (p < 0.05; one-way ANOVA and Bonferroni’s multiple comparison test) differences are for **125I-CYP binding**: PD and ΔC2-YFP.
* Significant (p < 0.05; one-way ANOVA and Bonferroni’s multiple comparison test) differences are for **isoproterenol competition**: PD and ΔC2-YFP.
* Significant (p < 0.05; one-way ANOVA and Bonferroni’s multiple comparison test) differences are for **isoproterenol competition**: PD and ΔAsn405.
* Significant (p < 0.05; one-way ANOVA and Bonferroni’s multiple comparison test) differences are for **isoproterenol competition**: PD and ΔC2-YFP.
* Significant (p < 0.05; one-way ANOVA and Bonferroni’s multiple comparison test) differences are for **isoproterenol competition**: ΔC2-PD and WT-YFP.
* Significant (p < 0.05; one-way ANOVA and Bonferroni’s multiple comparison test) differences are for **isoproterenol competition**: PD-C and WT-YFP.

FIGURE 2. Translocation of β-arrestin2 to the plasma membrane in response to receptor stimulation. HEK293 cells were transiently transfected with the indicated FLAG-tagged receptor, GRK2 and βArr2-YFP, and plated on coverslips. After addition of 10 μM isoproterenol, cells were observed by live confocal microscopy for 5 min. Shown are images before (ctrl) and after 2 min of agonist treatment (+iso). Longer treatment did not further change the appearance of the cells. The images are representative of at least three independent experiments. Bar, 10 μm.
**TABLE 2**

Kinetic constants for β-Ar2-YFP binding to various receptor mutants

| First stimulus | k (min⁻¹) | tᵰ (s) |
|----------------|----------|--------|
| WT             | 5.0 ± 2.2 | 8.3    |
| PD             | no binding |        |
| PD-N           | no binding |        |
| PD-C           | 9.7 ± 1.3 | 4.3    |
| ΔC2            | 3.8 ± 0.6 | 11.0   |
| ΔC2-PD         | ND       |        |
| ΔAsn405        | 4.0 ± 0.4 | 10.5   |

| Second stimulus | k (s⁻¹) | tᵰ (s) |
|-----------------|--------|--------|
| WT              | 0.64 ± 0.03 | 1.1   |
| ΔC2             | 0.91 ± 0.11 | 0.8   |
| ΔAsn405         | 0.68 ± 0.03 | 1.0   |

β-Ar2-YFP (Fig. 4B), indicating that mutation of the proximal serine/threonine residues did not completely prevent β-arrestin2 from interacting with the receptors. However, no direct interaction between YFP-tagged PD or PD-N mutant receptors and β-Ar2-CFP could be detected in FRET measurements under comparable conditions (Fig. 3, B and C), suggesting that either the amount of receptor–β-arrestin complex is too low to be detected by FRET or the observed β-arrestin2-mediated ratio F₅₃₅/F₄₈₀ (red). Data shown are representative of at least five independent experiments.

**TABLE 3**

Kinetic constants for β-Ar2-CFP binding to various receptor mutants

| First stimulus | k (min⁻¹) | tᵰ (s) |
|----------------|----------|--------|
| WT             | 0.64 ± 0.03 | 1.1   |
| ΔC2            | 0.91 ± 0.11 | 0.8   |
| ΔAsn405        | 0.68 ± 0.03 | 1.0   |

Arrestin-independent Role of β₂AR C-tail in Internalization

- The above data suggested that residues in the distal part of the β₂AR C terminus are not required for β-arrestin binding. To investigate whether this part of the receptor contributes to β-arrestin binding and receptor internalization at all, we constructed a truncated receptor mutant (ΔC2), in which the C terminus distal to leucine 381, containing the serine and threonine residues which were mutated in PD-C, was deleted (Fig. 1). As shown in Fig. 5, the ΔC2 receptor was fully capable to interact with β-arrestin2, as revealed by an agonist-dependent, quantitative translocation of β-Ar2-YFP to the plasma membrane (Fig. 5A) and a strong signal detected in the FRET assay (Fig. 5B). Evaluation of the binding curves suggested that the kinetics of β-arrestin2 binding to ΔC2 was similar to the wild-type receptor after the first stimulation (Table 2). If the receptor had already been prestimulated, β-arrestin2 bound significantly faster to the ΔC2 mutant than to the wild-type receptor (tᵰ of 1.1 s for wild-type receptor versus 0.8 s for ΔC2). These data suggest that the distal C terminus is dispensable for efficient β₂AR phosphorylation and slightly impedes β-arrestin2 binding to the phosphorylated receptor.

- **Phosphorylation of PD-C and ΔC2 Mutants**—To explore whether the PD-C and ΔC2 mutants showed altered phosphorylation by GRK2, we compared their phosphorylation state to that of the wild-type receptor in unstimulated and agonist-stimulated cells. Fig. 6A shows that, in unstimulated cells, all three receptors showed little phosphorylation, which could be clearly increased by treating the cells for 5 min with 1 µM isoproterenol. Quantitative analysis of repeated experiments revealed that there was no significant difference between the three receptors when the amount of agonist-stimulated phosphorylation was normalized to the basal phosphorylation (Fig. 6B).

- **Activity of the PD-C and ΔC2 Mutants**—To investigate a possible role for the distal receptor C terminus in receptor activation, we determined the cAMP production by WT, PD-C, and ΔC2 receptors in membranes prepared from transiently transfected HEK293 cells and compared them. Because constitutive activity also depends on the amount of receptor expression, we took care to compare only membrane preparations that contained similar amounts of receptor, as determined by 125I-CYP binding. Table 3 shows that both the PD-C and ΔC2 mutants
coupled to adenyl cyclase very much like wild-type receptor in response to increasing amounts of isoproterenol. While WT and PD-C mutant showed the same basal activity, basal activity of the H9004C2 mutant was increased but the difference did not reach statistical significance.

**β-Arrestin Interaction of the ΔC2 Mutant Requires Receptor Phosphorylation**—As shown above, binding of β-arrestin2 to the ΔC2 receptor is accelerated after repeated stimulation, when compared with binding after the first stimulus. This suggested that β-arrestin2 binding to ΔC2 still required receptor phosphorylation. To explore this, we constructed the H9004C2-PD mutant in which the proximal serine and threonine residues between residues 355 and 364 were mutated to alanine and glycine (Fig. 1), leading to a mutant receptor corresponding to a C-terminally truncated PD-N. Again, mutation of the proximal serine/threonine cluster resulted in a receptor in which β-arrestin2 binding was severely impaired. The H9004C2-PD receptor was unable to induce quantitative translocation of β-arrestin2 to the plasma membrane (Fig. 7A), and the FRET signal between YFP-tagged ΔC2 mutant and βAr2-CFP was dramatically reduced (Fig. 7B). These findings indicate that efficient β-arrestin2 binding still requires receptor phosphorylation at the proximal serine/threonine cluster, even when the distal C terminus is removed. However, in contrast to the full-length PD-N receptor, the ΔC2-PD mutant was able to recruit a small amount of β-arrestin2, which appeared as a distinct vesicular staining close to the plasma membrane after agonist treatment (Fig. 7A). Similarly, the FRET signal obtained with ΔC2-PD was strongly reduced, but not completely abolished (Fig. 7B); unfortunately, the data were too noisy to be evaluated by curve fit.
This result was rather unexpected, since the relative affinity of the activated receptor for β-arrestin2 very efficiently (Fig. 5). Therefore, additional elements seem to exist in the distal C terminus which are required for efficient internalization of the β2AR. Interestingly, internalization of the ΔC2 receptor could be fully rescued by overexpression of β-arrestin2 (Fig. 8B), which argues for a combined action of β-arrestin2 and the β2AR distal C terminus. This finding was corroborated by the internalization pattern of the ΔC2-PD receptor, in which both the proximal and distal parts of the C terminus are altered. Such a “double mutant” receptor was completely unable to internalize in an agonist-dependent manner. Importantly, its internalization was only poorly rescued by overexpression of β-arrestin2 (Fig. 8B) when compared with that of PD mutants with a full-length C terminus (Fig. 4B). To further map the site on the β2AR C terminus that aids in receptor internalization, the ΔAsn405 mutant was constructed. It lacks the last eight amino acids. This mutant is unable to internalize when co-expressed with β2AR C2 receptor and its expression is impaired in the wild-type receptor (Fig. 4). Therefore, additional elements seem to exist in the distal C terminus which are required for efficient internalization of the β2AR. Interestingly, internalization of the ΔC2 receptor could be fully rescued by overexpression of β-arrestin2 (Fig. 8B), which argues for a combined action of β-arrestin2 and the β2AR distal C terminus. This finding was corroborated by the internalization pattern of the ΔC2-PD receptor, in which both the proximal and distal parts of the C terminus are altered. Such a “double mutant” receptor was completely unable to internalize in an agonist-dependent manner. Importantly, its internalization was only poorly rescued by overexpression of β-arrestin2 (Fig. 8B) when compared with that of PD mutants with a full-length C terminus (Fig. 4B). To further map the site on the β2AR C terminus that aids in receptor internalization, the ΔAsn405 mutant was constructed. It lacks the last eight amino acids. This mutant is unable to internalize when co-expressed with β2AR C2 receptor and its expression is impaired in the wild-type receptor (Fig. 4). Therefore, additional elements seem to exist in the distal C terminus which are required for efficient internalization of the β2AR.

The Distal C Terminus Is Required for β-Arrestin2-dependent Receptor Internalization—Next we analyzed whether removal of the distal C terminus impaired (24% internalized receptors after 30 min), when compared with the wild-type receptor (32% internalized receptors). This result was rather unexpected, since the ΔC2 receptor bound β-arrestin2 very efficiently (Fig. 5). Therefore, additional elements seem to exist in the distal C terminus which are required for efficient internalization of the β2AR. Interestingly, internalization of the ΔC2 receptor could be fully rescued by overexpression of β-arrestin2 (Fig. 8B), which argues for a combined action of β-arrestin2 and the β2AR distal C terminus. This finding was corroborated by the internalization pattern of the ΔC2-PD receptor, in which both the proximal and distal parts of the C terminus are altered. Such a “double mutant” receptor was completely unable to internalize in an agonist-dependent manner. Importantly, its internalization was only poorly rescued by overexpression of β-arrestin2 (Fig. 8B) when compared with that of PD mutants with a full-length C terminus (Fig. 4B). To further map the site on the β2AR C terminus that aids in receptor internalization, the ΔAsn405 mutant was constructed. It lacks the last eight amino acids. This mutant displayed wild-type-like β-arrestin2 binding as determined by FRET (Table 2, t½ of 10.5 s after the first and t½ of 1.1 s after the second stimulus), but impaired receptor internalization (Fig. 8B).
Arrestin-independent Role of β2AR C-tail in Internalization

Internalization was induced by the addition of 1 μM isoproterenol for the times indicated. Cell surface receptors were quantified by [3H]CGP12,177 binding. In all experiments, receptor expression levels were determined in HEK293 cells transiently transfected with FLAG-tagged wild-type, ΔC2 or ΔC2-PD receptors, βArr2-YFP and GRK2. Data shown are means ± S.E. of at least three independent experiments, each performed in triplicate.

FIGURE 8. Role of the β2AR distal C terminus in receptor internalization. Internalization was induced by the addition of 1 μM isoproterenol for the times indicated. Cell surface receptors were quantified by [3H]CGP12,177 binding. In all experiments, receptor expression levels were determined in HEK293 cells transiently transfected with FLAG-tagged wild-type, ΔC2, ΔAsn405, or ΔC2-PD β2AR mutants. β2AR-YFP and GRK2. Data shown are means ± S.E. of at least three independent experiments, each performed in triplicate.

8A, 18% internalized receptors after 30 min). The ΔAsn405 mutant internalized even slightly less efficiently than the ΔC2 mutant. These data suggest that the last eight amino acids of the β2AR contain a domain that is involved in receptor internalization, but not arrestin binding.

DISCUSSION

In this report, we analyzed the role of the β2AR C terminus for β-arrestin binding and receptor internalization. We restricted our investigation to β-arrestin2, which has a higher affinity for the β2AR than β-arrestin1 (27).

It has been shown previously that simultaneous mutation of all serine/threonine residues in the β2AR C terminus prevents agonist-mediated receptor phosphorylation (22) and β-arrestin-mediated internalization (26). Here we show that not all of these residues are required for initial β-arrestin binding to the receptor. Previous research has demonstrated that phosphorylation of a cluster of four residues between Ser-355 and Ser-364 is essential for β-arrestin binding to the β2AR (6–8, 10). Consistent with these results, we show that mutation of only these proximal residues (the PD-N mutant) yields a receptor that is defective in agonist-induced β-arrestin2 binding, as evident from a variety of analyses: (1) loss of receptor-mediated plasma membrane translocation of βArr2-YFP (Fig. 2), (2) the loss of FRET between β2AR-YFP and βArr2-CFP (Fig. 3C) and (3) a loss of receptor internalization (Fig. 4A).

Mutation of the proximal serine/threonine cluster did not completely abolish the ability of the β2AR receptor to interact with β-arrestin2. The affinity of β-arrestin2 for an activated, but non-phosphorylated receptor seemed to be dramatically reduced, but not completely abolished. Using FRET, we observed a weak interaction between the activated ΔC2-PD mutant receptor and βArr2-YFP (Fig. 7). This could be explained in two ways. First, truncation of the C terminus in the ΔC2-PD mutant could have reduced the distance or improved the orientation of the two fluorophores compared with the situation in the full-length phosphorylation-deficient (PD and PD-N) mutants, resulting in higher sensitivity for detecting the receptor-arrestin complex. Alternatively, β-arrestin2 binding to the truncated receptor could be facilitated in comparison to the full-length receptor. Such a mechanism has been proposed for the D1 dopamine receptor (21). A D1 receptor in which all of the GRK phosphorylation sites had been removed by truncation, β-arrestin2 could still bind to the activated receptor, and desensitization was still observed. However, internalization of the truncated D1 mutant was dramatically impaired. In the β2AR, truncation of the receptor did not abolish the requirement for receptor phosphorylation, as β-arrestin2 binding to the ΔC2 mutant was much stronger than binding to the ΔC2-PD mutant (which presumably cannot be phosphorylated by GRKs; compare Fig. 7 to Fig. 5). Thus, phosphorylation of the proximal receptor C terminus is required for a high affinity interaction with β-arrestin2. If the β2AR had been phosphorylated by a first agonist stimulus, application of agonist for a second time caused faster binding of β-arrestin2 to the ΔC2 mutant than to the wild-type receptor (Table 2). This suggests that the distal C terminus slightly impeded access of β-arrestin2 to the rest of the receptor, even in a GRK-phosphorylated state.

The observed residual binding to the ΔC2-PD mutant was still agonist-dependent, which suggests that other parts of the receptor contain a β-arrestin2 binding site(s), which change their conformation upon agonist activation. An additional β-arrestin binding site in the β2AR has been identified in the second intracellular loop (20), and the third intracellular loop may also contribute to β-arrestin binding, as suggested from results with other receptors (13–18). This is in agreement with the currently accepted model of arrestin binding to G-protein-coupled receptors (11).

Recruitment of β-arrestins to activated G-protein-coupled receptors is known to be a prerequisite for receptor internalization (3). Accordingly we show that for those of our receptor mutants, which did not efficiently bind β-arrestin2 (PD, PD-N,
and ΔC2-PD), internalization was consistently severely impaired. However, it appears that effective recruitment of β-arrestins is not sufficient for efficient receptor internalization, because deletion of the distal C terminus generated a receptor (the ΔC2 mutant) that was impaired in internalization despite strong β-arrestin2 binding. Deletion of the last eight residues of the β2AR (ΔAsn405 mutant) was sufficient to reproduce the phenotype, suggesting that it is these amino acids that aid in internalization. Indeed, three of these amino acids are altered in the PD-C mutant which may explain the slightly reduced internalization of this mutant compared with wild-type (Fig. 4A, 35% internalized receptors after 30 min of stimulation, versus 47% wild-type receptors). Interestingly, overexpression of β-arrestin2 rescued the internalization-deficient phenotype of the ΔC2 mutant, i.e. elevated β-arrestin2 levels could compensate for the loss of the receptor distal C terminus.

Based on the evidence from the literature, we believe that the role of the distal β2AR C terminus is most likely to coordinate further protein-protein interactions aiding in the internalization process. Proteins that have been shown to bind to the β2AR include protein kinase A-anchoring proteins (AKAPs) (28, 29), the membrane fusion regulatory protein, N-ethylmaleimide-sensitive factor (NSF) (30), or the Na+/H+ exchanger regulatory factor (NHERF/EBP50) (31, 32). Both NSF and NHERF/EBP50 have been described to bind the C-terminal tail that is deleted in the ΔAsn405 mutant (30–32). Of particular interest, NSF can also interact with β-arrestin1, although the interaction between NSF and β-arrestin2 was not investigated (33). We suggest that the distal β2AR C terminus coordinates the interaction between β-arrestins and further proteins, thus aiding in the formation of a protein complex that is required for efficient internalization of the receptor. Our experiments do not address whether these interactions are required for efficient targeting of the receptor-arrestin complex into clathrin-coated pits or for the subsequent transformation of the pit into a clathrin-coated vesicle.

Why did overexpression of β-arrestin2 rescue the internalization defect of the PD and PD-N mutant (Fig. 4B) but was not efficient at rescuing the internalization defect of the ΔC2-PD mutant (Fig. 8B)? In the FRET assay, the receptor with the lowest affinity to β-arrestin2 is the PD mutant (compare Figs. 3 and 7), so the trivial explanation that the sensitivity of our FRET microscope is too low to detect low affinity receptor-arrestin interactions (34) seems unlikely. We hypothesize that upon overexpression of β-arrestin2, it can bind indirectly to the PD and PD-N mutants via another protein that interacts with the distal β2AR C terminus. If the interaction of this putative “bridging” protein with the β2AR was dependent on a free receptor C terminus, we would not be able to pick up this complex formation in the FRET assay. Internalization of the ΔC2-PD mutant cannot be rescued efficiently by overexpression of β-arrestin2 because the unknown arrestin binding partner is unable to interact with the receptor. This proposed mechanism might not be restricted to the β2AR: it has been reported that phosphorylation-deficient mutants of the δ-opioid receptor were able to internalize in a β-arrestin2-dependent manner (35).

In summary, the experiments reported in this report and our previous publication (4) can be explained by a model (11) in which β-arrestins first interact with agonist-activated, GRK-phosphorylated β2ARs. The phosphorylated residues required for β-arrestin binding are located in the proximal C terminus between Ser-355 and Ser-364. Receptor-bound β-arrestin2 then acts in concert with the distal receptor C terminus to allow efficient internalization.

Acknowledgments—We thank Susanna Cotecchia and Vsevolod Gurevich for providing cDNAs. We also thank Christian Dees for performing the ligand binding assays.

REFERENCES

1. Lohse, M. J. (1993) Biochim. Biophys. Acta 1179, 171–188
2. Lefkowitz, R. J., and Shenoy, S. K. (2005) Science 308, 512–517
3. Ferguson, S. S. G. (2001) Pharmacol. Rev. 53, 1–24
4. Krasel, C., Bünemann, M., Lorenz, K., and Lohse, M. J. (2005) J. Biol. Chem. 280, 9528–9535
5. Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 13796–13803
6. Hausdorff, W. P., Campbell, P. T., Ostrowski, I., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2979–2983
7. Seibold, A., January, B. G., Friedman, J., Hipkin, R. W., and Clark, R. B. (1998) J. Biol. Chem. 273, 7637–7642
8. Seibold, A., Williams, B., Huang, Z.-F., Friedman, J., Moore, R. H., Knoll, B. J., and Clark, R. B. (2000) Mol. Pharmacol. 58, 1162–1173
9. Trester-Zedlitz, M., Burlingame, A., Koblika, B., and von Zastrow, M. (2005) Biochemistry 44, 6133–6143
10. Vaughan, D. J., Millman, E. E., Godines, V., Friedman, J., Tran, T. M., Dai, W., Knoll, B. J., Clark, R. B., and Moore, R. H. (2006) J. Biol. Chem. 281, 7684–7692
11. Gurevich, V. V., and Gurevich, E. V. (2004) Trends Pharmacol. Sci. 25, 105–111
12. Kowoor, A., Celver, I., Abdryashitov, R. I., Chavkin, C., and Gurevich, V. V. (1999) J. Biol. Chem. 274, 6831–6834
13. Wu, G., Krapnick, J. I., Benovic, J. L., and Lanier, S. M. (1997) J. Biol. Chem. 272, 17836–17842
14. Gelber, E. I., Kroeze, W. K., Willins, D. L., Gray, J. A., Sinar, C. A., Hyde, E. G., Gurevich, V., Benovic, J., and Roth, B. L. (1999) J. Neurochem. 72, 2206–2214
15. Cen, B., Xiong, Y., Ma, L., and Pei, G. (2001) Mol. Pharmacol. 59, 758–764
16. Cen, B., Yu, Q., Guo, J., Wu, Y., Ling, K., Cheng, Z., Ma, L., and Pei, G. (2001) J. Neurochem. 76, 1887–1894
17. DeGraff, J. L., Gurevich, V. V., and Benovic, J. L. (2002) J. Biol. Chem. 277, 43247–43325
18. Wang, Q., and Limbird, L. E. (2002) J. Biol. Chem. 277, 50589–50596
19. Wu, N., Macion-Dazard, R., Nithianantham, S., Xu, Z., Hanson, S. M., Vishnietskii, S. A., Gurevich, V. V., Thibonnier, M., and Shoham, M. (2006) Mol. Pharmacol. 70, 249–258
20. Marion, S., Oakley, R. H., Kim, K. M., Caron, M. G., and Barak, L. S. (2006) J. Biol. Chem. 281, 2932–2938
21. Kim, O.-J., Gardner, B. R., Williams, D. B., Cabrera, D. M., Peters, J. D., Mak, C. C., Kim, K.-M., and Sibley, D. R. (2004) J. Biol. Chem. 279, 7999–8010
22. Bouvier, M., Hausdorff, W. P., De Blasi, A., O’Dowd, B. F., Koblika, B. K., Caron, M. G., and Lefkowitz, R. J. (1988) Nature 333, 370–373
23. Gabilondo, A. M., Krasel, C., and Lohse, M. J. (1996) Eur. J. Pharmacol. 307, 243–250
24. Dicker, F., Quitterer, U., Winstel, R., Honold, K., and Lohse, M. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5476–5481
25. Lorenz, K., Lohse, M. J., and Quitterer, U. (2003) Nature 426, 574–579
26. Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Ménard, L., and Caron, M. G. (1996) Science 271, 363–366
27. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) J. Biol. Chem. 275, 17201–17210
28. Shih, M., Lin, F., Scott, J. D., Wang, H. Y., and Malbon, C. C. (1999) J. Biol. Chem.
Arrestin-independent Role of β₂AR C-tail in Internalization

32. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) Nature 401, 286–290
33. McDonald, P. H., Cote, N. L., Lin, F. T., Premont, R. T., Pitcher, J. A., and Lefkowitz, R. J. (1999) J. Biol. Chem. 274, 10677–10680
34. Violin, J. D., Ren, X. R., and Lefkowitz, R. J. (2006) J. Biol. Chem. 281, 20577–20588
35. Zhang, X., Wang, F., Chen, X., Li, J., Xiang, B., Zhang, Y. Q., Li, B. M., and Ma, L. (2005) J. Neurochem. 95, 169–178