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Die Funktion der Helix 8 für die Regulation des Bradykinin B_2 Rezeptors

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Für Marion

1. Zusammenfassung

Der Bradykinin B_2 Rezeptor (B_2R) gehört zur Familie der G-Protein-gekoppelten Rezeptoren (GPCRs, G protein-coupled receptors), die mit fast 1000 Vertretern die größte Familie der Membranrezeptoren darstellen. GPCRs interagieren nach der Aktivierung mit heterotrimeren G-Proteinen und induzieren bei ihnen den Austausch von GDP zu GTP. Die aktivierten G-Protein Untereinheiten (Gα-GTP und Gβγ) können anschließend mit Effektorproteinen wechselwirken und so verschiedenste Vorgänge der Zellphysiologie regulieren. Der B_2R wird nach der G-Protein-Aktivierung durch G-Protein-gekoppelte Rezeptorkinasen (GRKs) und „second messenger"-Kinasen phosphoryliert und interagiert mit β-Arrestinen, was die weitere G-Protein Anlagerung verhindert und die Internalisierung des Rezeptors über sogenannte „Clathrin-coated-pits" einleitet. Strukturell werden für den B_2R, wie für alle GPCRs, sieben Transmembrandomänen, drei extrazelluläre und drei intrazelluläre Schleifen sowie ein intrazellulär lokalisierter C-Terminus postuliert (Abb.1). Neben den sieben helikalen Transmembrandomänen existiert noch eine weitere α-Helix (Helix 8), die im Anschluss an die siebte Transmembrandomäne zytosolisch und parallel zur Plasmamembran angeordnet ist und einen Teil des C-Terminus darstellt (Abb.1).

In der vorliegenden Arbeit konnten wir zeigen, dass die Störung der Sekundärstruktur der Helix 8 durch Deletion des C-Terminus oder durch die Mutation einer zentralen Aminosäure (Lys-315) zu einem Prolin (Helixbrecher) zu einer starken Abnahme der Oberflächenexpression des B_2R führte. Der fehlerhafte Transport des Rezeptors zur Zellmembran konnte durch die Zugabe eines membrangängigen „small-molecule" B_2R Antagonisten (JSM10292) korrigiert werden. Folglich übernimmt die Helix 8 eine wichtige Aufgabe bei der Stabilisierung der für den Transport an die Plasmamembran notwendigen Rezeptorkonformation, die durch einen entsprechenden Antagonisten übernommen werden kann. In der vorliegenden Arbeit wird außerdem gezeigt, dass die strukturelle Integrität der Helix 8 und nicht, wie bisher allgemein angenommen, vor allem der C-Terminus (mit den Phosphorylierungsstellen) für die Internalisierung des B_2R und seiner Interaktion mit GRK2/3 und β-Arrestin2 unerlässlich ist. Der C-Terminus ist jedoch für die stabile Interaktion von β-Arrestin1 mit dem B_2R erforderlich. Obwohl die G-Protein-Aktivierung nach unseren Ergebnissen anscheinend auch über die Helix 8 vermittelt wird, konnten wir durch ein „alanine-screening" der drei intrazellulären Schleifen belegen, dass wie erwartet vor allem hier die Rezeptordomänen liegen, die für eine produktive Interaktion mit den G-Proteinen entscheidend sind. Für den Prozess der Rezeptor-Internalisierung ist der Beitrag der intrazellulären Schleifen im Gegensatz zur Rolle der Helix 8 vermutlich nur von untergeordneter Bedeutung.

Zusammenfassend zeigen die Ergebnisse der Arbeit erstmalig, dass die Helix 8 eine strukturelle Einheit darstellt, deren Integrität neben ihrer Funktion für den Transport des B_2Rs zur Plasmamembran eine entscheidende Rolle für die Aktivierung und die Rekrutierung von intrazellulären Proteinen spielt, die an der Signaltransduktion und der Internalisierung des Rezeptors beteiligt sind. Durch die Generierung von Rezeptorchimären aus B_2R und anderen GPCRs konnten wir darüber hinaus zeigen,

dass die Helix 8 auch bei anderen GPCRs eine entsprechend tragende Funktion bei Transport, Signaltransduktion und Internalisierung hat.

2. Summary

The bradykinin B2 Receptor (B_2R) belongs to the family of G protein-coupled receptors (GPCRs), which comprises the largest class of membrane receptors (approx. 1000 members). Upon activation GPCRs bind to heterotrimeric G proteins and cause their exchange of GDP for GTP. The activated G protein subunits (Gα-GTP und Gβγ) can then interact with downstream effectors to modulate various aspects of cellular signaling. Subsequent to G protein activation the B_2R becomes phosphorylated through G protein-coupled receptor kinases (GRKs) and second messenger kinases and interacts with β-arrestins, thereby preventing further G protein binding and promoting receptor internerlization via clathrin-coated-pits. Like all other GPCRs, the B_2R exhibits a seven transmembrane helix topology, with three extracellular and three intracellular loops as well as a cytosolic C-terminus. Moreover, the B_2R shows an intracellular α-helix (Helix 8) that is linked to transmembrane helix 7 and placed parallel to the plasmamembrane being a part of the C-terminus.

We show here that disruption of helix 8 in the B_2R by either C-terminal deletion or just by mutation of a central amino acid (lys-315) to a helix-breaking proline resulted in strong reduction of surface expression. Interestingly, this malfunction could be overcome by addition of the membrane-permeable B_2R antagonist JSM10292, suggesting that helix 8 has a general role for conformational stabilization that can be accounted for by an appropriate antagonist. Intriguingly, an intact helix 8, but not the C-terminus with its phosphorylation sites, was indispensable for receptor internalization and for interaction of the B_2R with GRK2/3 and β-arrestin2. Recruitment of β-arrestin1, however, required the presence of the C-terminus. Although G protein activation is mediated in part through the action of helix 8 we could also show the special importance of the intracellular loops for the productive interaction with G proteins by using an alanine-screening approach. However, in contrast to helix 8 the intracellular loops are obviously not essential for receptor internalization.

Taken together, our results demonstrate for the first time that helix 8 of the B_2R plays a crucial role not only in efficient trafficking to the plasma membrane but also for the interaction and activation of intracellular proteins that promote signaling and internalization. Additional data obtained with chimera of B_2R with other GPCRs suggest that helix 8 might have similar functions in other GPCRs, too.

3. Grundlegendes zur Struktur und molekularen Regulation von GPCRs am Beispiel des B_2 Bradykinin Rezeptors

G-Protein-gekoppelte Rezeptoren (GPCRs) stellen mit fast 1000 Vertretern die größte Familie der Membranrezeptoren dar und sind maßgeblich an der Regulation von vielen zellphysiologischen Vorgängen beteiligt. Strukturell zeichnen sich GPCRs durch sieben Transmembranhelices (TM 1-7) aus, die durch drei extrazelluläre (ECL 1-3; extracellular loops) und drei intrazelluläre (ICL 1-3; intracellular loops) Schleifen miteinander verbunden sind (Palczewski *et al.*, 2000) (Abb.1). Die Aufklärung der Struktur mehrerer GPCRs hat ergeben, dass noch eine weitere α-Helix (Helix 8) existiert, die im Anschluss an die hoch konservierte NPXXY [1] -Sequenz der siebten Transmembranhelix (TM7) parallel zur Plasmamembran angeordnet ist und einen Teil des C-Terminus darstellt (Huynh *et al.*, 2009) (Abb.1). Diese α-Helix zeichnet sich allgemein durch einen ausgeprägt amphipathischen Charakter aus, wobei der hydrophobe Teil der lipophilen Zellmembran zugewandt ist und die hydrophile Seite in Richtung Zytosol zeigt. Bei vielen GPCRs, so auch im B_2R, wird die Helix 8 an ihrem Ende durch acylierte Cysteine in der Membran verankert (z.B. durch Palmitinsäure) und somit in Position gehalten (Abb.1). Bei Säugetieren unterscheidet man drei verschiedene Familien von G-Protein-gekoppelten Rezeptoren (A, B und C), die sich hauptsächlich in der Länge einzelner Domänen (Schleifen, N- und C-Terminus) sowie in der speziesübergreifenden Konservierung einzelner Aminosäuren unterscheiden (Gether, 2000).

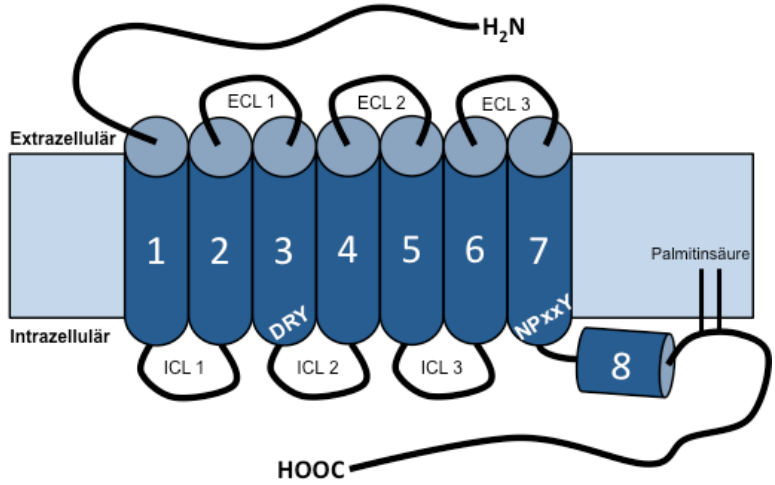

Abb.1. Schematische Darstellung der Struktur eines typischen G-Protein-gekoppelten Rezeptors (Familie A). Dargestellt sind die sieben Transmembrandomänen (1-7) sowie die drei extrazellulären (ECL 1-3) und drei intrazellulären Schleifen (ICL 1-3). Die Helix 8 liegt horizontal zur Plasmamembran und wird durch mit Palmitinsäure (schwarze Balken) acylierte Cysteine in der Plasmamembran fixiert. Am Ende von TM 3 und TM 7 befinden sich die hoch konservierte DRY- bzw. NPxxY-Sequenz.

Der B_2 Bradykinin Rezeptor (B_2R) gehört zur Familie A der GPCRs und wird ubiquitär in verschiedenen Zellen (z.B. Endothelzellen, glatte Muskelzellen, Fibroblasten, Nervenzellen und verschiedenen Tumorzellen) exprimiert (Leeb-Lundberg *et al.*, 2005). Er ist ein wesentlicher Bestandteil des Kallikrein-Kinin-Systems und dabei insbesondere an der Regulation vaskulärer und inflammatorischer Prozesse beteiligt. Kallikreine sind Serinproteasen die aus ihrem Substrat, den Kininogenen, die von der Leber synthetisiert und in den Blutkreislauf sezerniert werden durch limitierte Proteolyse die Kinine (Bradykinin (BK) und Kallidin (KD)) freisetzen. Kallidin

[1] X: eine beliebige proteinogene Aminosäure

unterscheidet sich vom Nonapeptid Bradykinin lediglich durch ein zusätzliches N-terminales Lysin weswegen es auch als Lys-BK bezeichnet wird. Diese beiden Peptide binden spezifisch an den B_2 Rezeptor und lösen in den Zielzellen die entsprechenden biologischen Effekte aus (z.B. Gefäßerweiterung, Erhöhung der Gefäßpermeabilität und Schmerzweiterleitung).

Nach der Aktivierung durch Hormone (z.B. Bradykinin beim B_2R), Neurotransmitter oder physikalischen Reizen wird bei GPCRs eine Konformationsänderung ausgelöst die zur intrazellulären Bindung von heterotrimeren G-Proteinen führt. Aktivierte GPCRs sind Guanin-Nukleotid-Austauschfaktoren für die Gα-Untereinheit der G-Proteine und bewirken bei diesen den Austausch von GDP zu GTP (Abb. 2, Aktivierung & Signaltransduktion), was zu ihrer Aktivierung führt. Die aktiven G-Protein Untereinheiten (Gα-GTP und Gβγ) können anschließend mit Effektorproteinen (z.B. Adenylatzyklase oder Phospholipasen) interagieren und so verschiedenste Vorgänge der Zellphysiologie modulieren.

Liganden-aktivierte GPCRs binden neben G-Proteinen auch sogenannte G-Protein-gekoppelte Rezeptorkinasen (GRKs) und werden von diesen phosphoryliert (Reiter *et al.*, 2006). Es sind insgesamt sieben verschiedene Subtypen von GRKs bekannt (GRK 1-7), die in verschiedenen Geweben exprimiert werden und unterschiedliche Präferenzen für bestimmte GPCRs aufweisen. Die Phosphorylierung von GPCRs durch GRKs führt zu einer verstärkten Interaktion mit Arrestinen - eine Gruppe von Gerüstproteinen (Arrestin 1-4), die ebenfalls in verschiedenen Geweben exprimiert werden. Die im Gegensatz zu Arrestin1 und 4 (nur in Netzhautzellen) ubiquitär auftretenden Arrestine 2 und 3 werden auch als β-Arrestin 1 und 2 bezeichnet, weil ihre Funktion erstmals im Zusammenhang mit den β-Adrenergen Rezeptoren aufgeklärt wurde. Im Folgenden soll ausschließlich die letztere Bezeichnung Verwendung finden.
Die Interaktion von GPCRs mit β-Arrestinen verhindert zum einen die weitere Anlagerung und Aktivierung von G-Proteinen und führt zum anderen bei vielen GPCRs zu einer β-Arrestin-vermittelten Internalisierung (Abb. 2) des Rezeptor in sogenannten „clathrin-coated-pits“ (Reiter *et al.*, 2006). Dieser Prozess der Signalabschwächung, der auch als Desensitisierung bezeichnet wird, spielt eine wichtige Rolle bei der Feinregulation der Aktivität von GPCRs. Forschungsergebnisse der letzten Jahre zeigen, dass die vom Rezeptor rekrutierten β-Arrestine neben ihrer Funktion als Adaptorproteine für die Internalisierung des Rezeptors auch als Gerüstproteine zur Aktivierung G-Protein-unabhängiger Signalwege wie z.B. dem MAP-Kinase-Weg (MAP, mitogen-activated protein) dienen.
Nach der Internalisierung werden die Rezeptorproteine entweder in den Lysosomen der Zelle abgebaut (Degradation) oder zurück an die Zelloberfläche transportiert, um erneut für (weitere) Stimulationen zur Verfügung zu stehen (Recycling) (Abb. 2). Wie dies genau gesteuert wird und wodurch das weitere Schicksal eines Rezeptors entschieden wird, ist im Detail noch nicht geklärt.

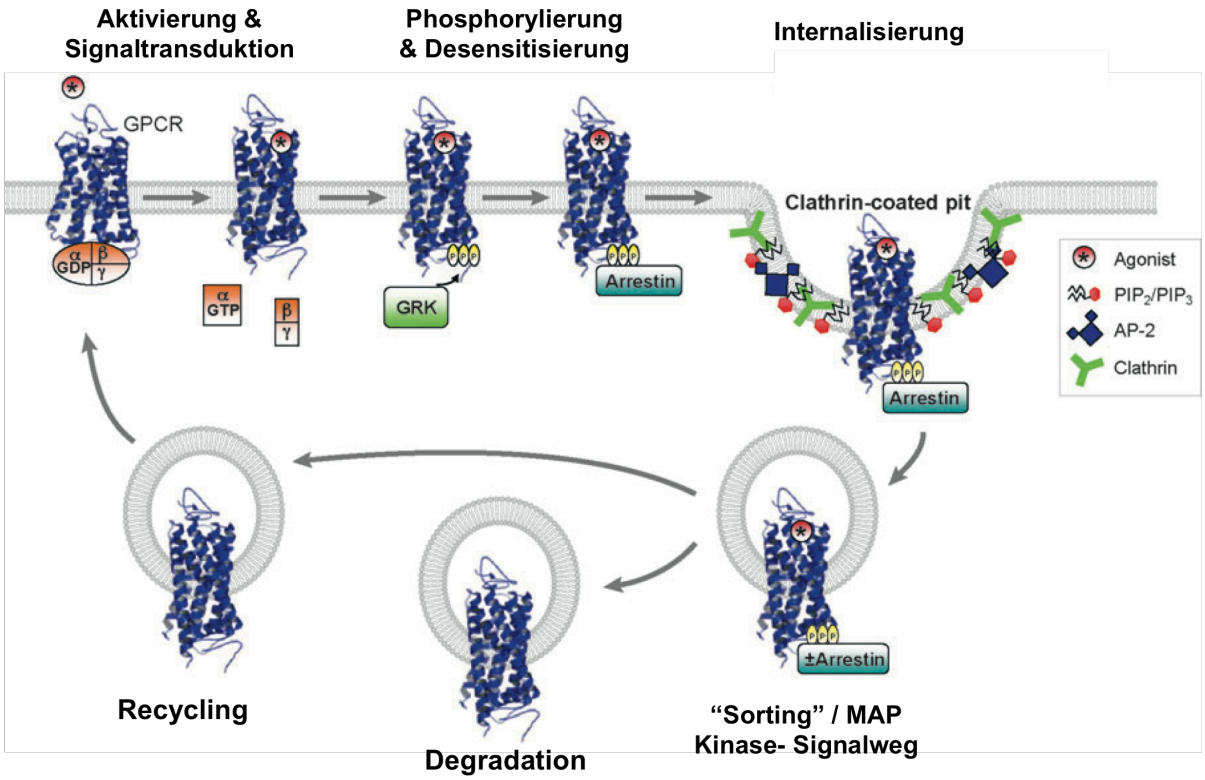

Abb2. Klassischer Regulationsmechanismus von GPCRs, der so auch für den B_2 Bradykinin Rezeptor gültig ist. PIP_2/PIP_3: Phosphatidylinositolbisphosphat/-trisphosphat; AP-2: Adaptorprotein-2 (modifiziert nach (Moore *et al.*, 2007)).

3.1. Die Rolle der intrazellulären Domänen für die Regulation des B_2 Bradykinin Rezeptors

Obwohl die grundlegenden Mechanismen der Signaltransduktion und der Regulation des B_2R weitestgehend untersucht wurden, ist die Information zu den molekularen und strukturellen Wechselwirkungen zwischen Rezeptor und den intrazellulären Kopplungsproteinen noch ziemlich begrenzt. Welche intrazellulären Elemente des B_2R (ICLs, Helix 8, C-Terminus) hierbei eine Schlüsselrolle als Interaktionspartner für die G-Proteine, die GRKs und die β-Arrestine einnehmen, ist noch nicht geklärt.

3.1.1. Die Funktion der Helix 8 für die Rezeptor-Oberflächenexpression

Für Rhodopsin sowie für den muskarinischen M_3 Acetylcholin-Rezeptor konnten als Folge der Aktivierung Konformationsänderungen mit einer deutlichen räumlichen Verschiebung der Helix 8 gezeigt werden (Altenbach *et al.*, 2001; Li *et al.*, 2007). Diese Ergebnisse legen den Schluss nahe, dass diese Helix ein mögliches Schlüsselelement für die Weiterleitung von Informationen in die Zelle ist. Um die genaue Funktion des C-Terminus aber insbesondere der Helix 8 für die Regulation des B_2R zu entschlüsseln (Publikation A) wurden im Rahmen der Doktorarbeit mehrere Rezeptormutanten mit sukzessiv verkürztem C-Terminus in HEK (human embryonic kidney) 293 Zellen exprimiert. Zusätzlich generierten wir eine Mutante bei der die strukturelle Integrität der Helix 8 gestört wurde, indem eine zentrale Aminosäure (Lys-315) innerhalb der Helix 8-Sequenz gegen den Helixbrecher

Prolin ausgetauscht wurde. Mithilfe des gelb fluoreszierenden Proteins (YFP, yellow fluorescent protein) das erstmalig am N-Terminus des B_2R angefügt wurde, um die Funktion des C-Terminus nicht zu beeinträchtigen, konnte gezeigt werden, dass alle Mutationen oder Deletionen, welche die Struktur der Helix 8 (zer)störten, zu einer starken intrazellulären Akkumulation der YFP-B_2R-Fusionskonstrukte führten. Gleichzeitig wurden diese Mutanten nur unvollständig glykosyliert, was vermutlich für ihr Verbleiben im endoplasmatischen Retikulum (ER) verantwortlich ist. Die Zurückhaltung von GPCRs durch das Qualitätskontrollsystem des ER nach Mutation oder Deletion der entsprechenden Helix 8-Sequenzen kann entweder durch die unkorrekte Faltung des Rezeptorproteins (Thielen *et al.*, 2005; Yasuda *et al.*, 2009) oder das Fehlen von spezifischen Sequenzmotiven (Bermak *et al.*, 2001) ausgelöst werden. Wir konnten zeigen, dass der verminderte Transport an die Plasmamembran durch die Anwendung eines membrangängigen „small-molecule“ B_2R Antagonisten (JSM10292) korrigiert werden konnte. Daher gehen wir davon aus dass, die Helix 8 eine wichtige Aufgabe für die Stabilisierung der für den Transport an die Zellmembran notwendigen Rezeptorkonformation hat. Die Stabilisierung dieser Konformation kann offensichtlich auch durch einen entsprechenden Antagonisten, der dabei als sogenanntes „pharmakologisches Chaperon“ wirkt, übernommen werden.

3.1.2. Die Helix 8 als Vermittlerin der Rezeptor-Internalisierung

Wie oben bereits erwähnt, werden GPCRs spezifisch von GPCR Kinasen (GRKs) phosphoryliert. Ein wesentlicher Unterschied zwischen GRKs und anderen Kinasen ist die Tatsache, dass erstere keine genau definierten Konsensus-Sequenzen erkennen, jedoch unmittelbar vom Funktionszustand des Rezeptors abhängig sind: GRKs phosphorylieren ausschließlich liganden-aktivierte Rezeptoren. Obwohl die Zielsequenzen für die Phosphorylierung (Serine und Threonine im C-Terminus) beim B_2R bereits identifiziert wurden (Blaukat *et al.*, 2001), war bisher noch nichts über die anderen Rezeptorinteraktionsstellen für GRKs und ihre Spezifität für den B_2R bekannt.

Um die Spezifität der verschiedenen GRKs für den B_2R zu identifizieren, untersuchten wir den Einfluss der Überexpression von GRK 2-6 auf die Hemmung des MAP-Kinase-Signalweges (ERK1/2 Phosphorylierung) nach Stimulation mit dem Agonisten Bradykinin. Die ERK1/2 Phosphorylierung konnte nur durch Überexpression von GRK2 und GRK3 signifikant gehemmt werden. GRK1 und GRK7 wurden hierbei nicht untersucht, da sie ausschließlich in Netzhautzellen vorkommen und daher keine physiologische Relevanz für die Regulation des B_2R besitzen. Neben der Spezifität des B_2R für GRK2 und 3 konnten wir durch Co-Immunopräzipitation zeigen, dass die Interaktion mit diesen GRKs hauptsächlich von der Helix 8 und nicht vom C-Terminus und dessen Phosphorylierungsstellen bestimmt wird.

Die spezifische Phosphorylierung des B_2R galt bisher als wesentliche Voraussetzung für die Interaktion mit den β-Arrestinen und die anschließende Rezeptorinternalisierung. Außerhalb der Netzhaut gibt es zwei verschiedene β-Arrestin Subtypen (β-Arrestin1 und 2). Für den B_2R wurden

bisher jedoch keine funktionellen Unterschiede dieser beiden Formen beschrieben. In der vorliegenden Arbeit konnte gezeigt werden, dass die Rezeptorinternalisierung I: unabhängig von der Präsenz des C-Terminus II: subtyp-spezifisch durch β-Arrestin2 und III: maßgeblich durch die Helix 8 vermittelt wird. β-Arrestin1 kann unter bestimmten Umständen (Überexpression, siRNA-knockdown von β-Arrestin2) die Funktion von β-Arrestin2 für die Internalisierung des B_2R übernehmen, spielt aber unter „quasi-physiologischen" Bedingungen in HEK 293 Zellen eine untergeordnete Rolle für diesen Prozess. Anders als für β-Arrestin2 erfordert die stabile Bindung von β-Arrestin1 an den B_2R zwingend die Phosphorylierung des C-Terminus.

Mithilfe verschiedener Rezeptorchimären, bestehend aus dem B_2R und der Helix 8 und/oder dem C-Terminus verschiedener GPCRs (PAR 2, β_2-AR, AT_1R, B_1R)[2], konnten wir außerdem zeigen, dass die Helix 8 nur in ihrer Rezeptor-spezifischen Umgebung die Funktion als Vermittlerin der Rezeptorinternalisierung erfüllen kann. Der restliche C-Terminus hingegen war in diesem Zusammenhang beliebig austauschbar und funktionierte auch unabhängig von seinem eigentlichen Rezeptor-Grundgerüst.

3.1.3. Die Funktion der intrazellulären Domänen für die G-Protein Aktivierung

Die Tatsache, dass alle Helix 8-Mutanten in der Lage waren das G-Protein zu aktivieren, wenngleich mit einer starken Erhöhung der EC_{50}-Werte, bestätigte unsere Ergebnisse, dass auch andere intrazelluläre Bereiche außerhalb der Helix 8 als Interaktionsstellen für das G-Protein fungieren. Mithilfe eines „alanine-screenings" der drei intrazellulären Schleifen (z.T. als Gruppen von 3-5 Aminosäuren, z.T als Punktmutationen) und durch die anschließende Bestimmung der Inositolphosphat-Freisetzung (als Maß für die G-Protein-Aktivierung) konnten wir entsprechende Bereiche identifizieren (Publikation B). Hierbei waren bestimmte Sequenzen der dritten Schleife für die Aufrechterhaltung des inaktiven Grundzustands des Rezeptors wichtig, da die entsprechenden Alanin-Mutationen eine „semi-aktive" Konformation induzierten, die auch durch die Zugabe von neutralen Antagonisten aktiviert werden konnte. Mutationen innerhalb der zweiten Schleife verursachten eine Abnahme der Inositolphosphat-Freisetzung und erwiesen sich deshalb als wichtige Determinanten für die produktive Interaktion mit dem G-Protein. Im Gegensatz zur Helix 8 war die Bedeutung der intrazellulären Schleifen für den Prozess der Rezeptor-Internalisierung allerdings gering.

Die hoch konservierte DRY-Sequenz des B_2R (siehe Abb.1), die sich durch alle Klassen der GPCRs zieht spielt eine wichtige Rolle für die Aufrechterhaltung des inaktiven Rezeptorzustands (Rovati *et al.*, 2007). Vermutlich koordiniert sie intramolekulare Bindungen, die nach Ligandenbindung aufgebrochen werden, um eine entsprechende Änderung zur aktiven Konformation zu ermöglichen. Eine von uns generierte Alanin-Dreifachmutante (DRY→AAA), wurde nicht an der Oberfläche

[2] PAR 2, Protease-aktivierter Rezeptor 2; β_2-AR, β_2-Adrenerger Rezeptor; AT_1R, Angiotensin II Typ 1 Rezeptor; B_1R, Bradykinin B_1 Rezeptor.

exprimiert, weshalb ihre Funktion für die G-Protein-Kopplung nicht weiter untersucht werden konnte. Durch eine für die vorliegende Arbeit etablierte Deglykosylierungs- und Immunoblot-Methode konnte gezeigt werden, dass diese Mutante zumindest in HEK 293 Zellen unvollständig glykosyliert wird und wahrscheinlich deshalb von der Qualitätskontrolle im ER zurückgehalten wird.
Die Interpretation aller Daten, legt einen Mechanismus nahe, bei dem die Konformationsänderung des B_2R nach Bindung eines Agonisten eine Bewegung der Helix 8 einschließt, die zu einer Freilegung von intrazellulären Rezeptorelementen führt, die dem G-Protein vor der Aktivierung noch nicht ausreichend zugänglich sind. Die vor kurzem aufgeklärte Struktur eines aktivierten β_2-Adrenergen Rezeptors mit gebundenem G-Protein $G\alpha_s$ zeigt allerdings keinerlei Berührung zwischen der Helix 8 des Rezeptors und dem G-Protein (Rasmussen *et al.*, 2011). Da diese Struktur aber gewissermaßen den Endzustand darstellt, schließt dies eine Interaktion der beiden Elemente während des Aktivierungsprozesses nicht unbedingt aus.

Zusammenfassend zeigen unsere Ergebnisse, dass die Helix 8 eine strukturelle Einheit darstellt, deren Integrität neben dem Transport des Rezeptorproteins zur Plasmamembran eine entscheidende Rolle für die Aktivierung und die Bindung von intrazellulären Proteinen spielt, die an der Signaltransduktion und der Sequestrierung des B_2R beteiligt sind. Ob die Helix 8 hierbei als direkter Interaktionspartner für die angesprochenen Proteine dient und/oder indirekt über die Stabilisierung der Gesamtrezeptorkonformation wirkt, muss durch zukünftige Experimente geklärt werden. Durch die Generierung von Rezeptorchimären aus dem B_2R und verschiedenen GPCRs konnten wir jedoch zeigen, dass entsprechende Funktionen der Helix 8 auf für andere GPCRs gelten.

4. Medizinische Bedeutung der Arbeit

Derzeit entfalten ca. 50% aller auf dem Markt befindlichen Arzneistoffe ihre Wirkung direkt oder indirekt über GPCRs. Dennoch sind unsere Kenntnisse der molekularen Grundlagen für die Regulation von GPCRs immer noch begrenzt. Um pharmakologisch noch gezielter in die Signaltransduktion und damit in die Zellfunktion eingreifen zu können, bedarf es der Aufklärung von Unterschieden und Gemeinsamkeiten der molekularen Regulationsmechanismen dieser wichtigen Klasse von Membranrezeptoren. Von besonderer Bedeutung ist dabei die Beteiligung der verschiedenen GRK- und Arrestin-Subtypen, die diese Unterschiede mitbestimmen. Genauere Informationen zu den molekularen Interaktionsstellen und Sequenzmotiven des Rezeptors, die nach der Ligandenstimulation als Kontaktstelle für das G-Protein oder der GRKs und β-Arrestine dienen, wären von großem Nutzen. Die Bedeutung entsprechender Erkenntnisse zur Entwicklung neuer Arzneistoffklassen für GPCRs zeigt sich darin, dass insbesondere die GRKs und β-Arrestine an der Regulation der Anzahl von Rezeptormolekülen an der Zelloberfläche durch Prozesse wie Internalisierung, Down-Regulation und Recycling beteiligt sind und damit wesentlich über die Dauer und Stärke der Signaltransduktion entscheiden. Die Idee verschiedene Signalwege gezielt, d. h.

unabhängig voneinander, pharmakologisch beeinflussen zu können, führte in den letzten Jahren verstärkt zu Bemühungen, Rezeptor(ant)agonisten („biased-(ant)agonists“) zu entwickeln, die gezielt den G-Protein- oder den β-Arrestin-vermittelten Signalweg (MAP-Kinase-Weg) aktivieren oder inhibieren (Reiter *et al.*, 2012).
Im Zusammenhang mit den hier präsentierten Ergebnissen ist es von großem Interesse, dass jüngst kleine chemische Moleküle identifiziert wurden, die an die Helix 8 binden und so allosterisch die Regulation von GPCRs und deren physiologische Funktionen beeinflussen können (Andrews *et al.*, 2008; Dowal *et al.*, 2011). Solche Moleküle könnten neben den klassischen orthosterischen Liganden einen neuen Ansatz zur Entwicklung spezifischerer Arzneistoffe darstellen.

Die Inaktivierung bzw. der Abbau von Bradykinin erfolgt über das Angiotensin converting enzyme (ACE), wodurch das Kallikrein-Kinin-System mit dem Renin-Angiotensin-Aldosteron System (RAAS) vernetzt ist. Ursprünglich wurden die kardio- und renoprotektiven Wirkungen der ACE-Hemmer (z.B. Captopril, Ramipril) v.a. mit der Hemmung der Umwandlung von Angiotensin I zum stark vasokonstriktorisch und damit blutdrucksteigernd wirkenden Angiotensin II erklärt. Mittlerweile ist bekannt, dass diese Effekte auch durch den verminderten Abbau von Bradykinin und der damit verbundenen Gefäßentlastung mitbestimmt werden (Alhenc-Gelas *et al.*, 2011). Die den Kininen hier zugeschriebenen kardio- und renoprotektiven Effekte stellen darüber hinaus einen vielversprechenden Ansatzpunkt für die Entwicklung spezifischer B_2R Agonisten zur Behandlung von Herzkreislauferkrankungen und Diabetes dar. Um solche Arzneistoffe in die klinische Entwicklung zu bringen, sind genauere Kenntnisse über die Feinabstimmung der B_2R Regulation notwendig, die u.a. wie in der vorliegenden Arbeit gezeigt anhand von in-vitro Zellmodellen gewonnen werden können.
Das hereditäre Angioödem, eine seltene Erbkrankheit, die mit einer plötzlich auftretenden, Bradykinin-vermittelten lebensbedrohlichen Erweiterung der Blutgefäße einhergeht, wird bereits durch Anwendung eines spezifischen B_2R-Antagonisten (Icatibant) behandelt, was die medizinische Relevanz des besprochenen Themengebiets zusätzlich verdeutlicht (Greve *et al.*, 2011).

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7. Lebenslauf

8. Publikationen zur kumulativen Dissertation (A und B)

8.1. Helix 8 plays a crucial role in bradykinin B_2 receptor trafficking and signaling (A)

8.2. Alanine screening of the intracellular loops of the human bradykinin B_2 receptor – effects on receptor maintenance, G protein activation and internalization (B)

A

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Helix 8 Plays a Crucial Role in Bradykinin B_2 Receptor Trafficking and Signaling[S]

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Upon activation the human bradykinin B_2 receptor (B_2R) acts as guanine nucleotide exchange factor for the G proteins $G_{q/11}$ and G_i. Thereafter, it gets phosphorylated by G protein-coupled receptor kinases (GRKs) and recruits β-arrestins, which block further G protein activation and promote B_2R internalization via clathrin-coated pits. As for most G protein-coupled receptors of family A, an intracellular helix 8 after transmembrane domain 7 is also predicted for the B_2R. We show here that disruption of helix 8 in the B_2R by either C-terminal truncation or just by mutation of a central amino acid (Lys-315) to a helix-breaking proline resulted in strong reduction of surface expression. Interestingly, this malfunction could be overcome by the addition of the membrane-permeable B_2R antagonist JSM10292, suggesting that helix 8 has a general role for conformational stabilization that can be accounted for by an appropriate antagonist. Intriguingly, an intact helix 8, but not the C terminus with its phosphorylation sites, was indispensable for receptor sequestration and for interaction of the B_2R with GRK2/3 and β-arrestin2 as shown by co-immunoprecipitation. Recruitment of β-arrestin1, however, required the presence of the C terminus. Taken together, our results demonstrate that helix 8 of the B_2R plays a crucial role not only in efficient trafficking to the plasma membrane or the activation of G proteins but also for the interaction of the B_2R with GRK2/3 and β-arrestins. Additional data obtained with chimera of B_2R with other G protein-coupled receptors of family A suggest that helix 8 might have similar functions in other GPCRs as well.

The human bradykinin B_2 receptor (B_2R)[2] belongs to the family A (rhodopsin/β-adrenergic-like) of G protein-coupled receptors (GPCRs). The B_2R is ubiquitously expressed in many cells and tissues, and its activation results in a variety of physiological effects that range from vasodilatation and increased vascular permeability to hyperalgesia and natriuresis (1). Recent studies with B_2R knock-out mice also point to a protective role of the B_2R in the process of aging and in diabetes (2). After extracellular binding of its endogenous agonists, of the nona-peptide bradykinin (BK), or of kallidin (Lys-BK), the B_2R undergoes conformational changes that turn it into a guanine nucleotide exchange factor for the G proteins $G_{q/11}$ and G_i, thus leading to the activation of G protein-dependent signaling cascades. Among other events, this results in phosphatidylinositol hydrolysis and activation of MAPK pathways. As reported for many GPCRs, desensitization of the B_2R comes along with phosphorylation of serine/threonine residues in its C terminus by G protein-coupled receptor kinases (GRKs) or second messenger kinases as well as recruitment of β-arrestins and ends with the sequestration of the receptor into intracellular compartments (1). Upon short term stimulation the receptor gets recycled to the plasma membrane, whereas long term stimulation leads to the down-regulation of the receptor (3–5). Currently, only limited information is available on the molecular regulation of the B_2R interactions with the G proteins, GRKs, or arrestins. Thus, it is not clear whether the same or different domains of the B_2R convey the active state of the receptor and are involved in coupling of the various signaling proteins.

As a typical GPCR of family A, the B_2R contains the NP*XX*Y sequence at the end of helix 7 and most likely the intracellular helix 8, linked at the N terminus to the NP*XX*Y sequence by a four-amino acid spacer and anchored at its C terminus through palmitoylated cysteines to the inner leaflet of the plasma membrane. Helix 8 was first identified in the crystal structure of bovine rhodopsin (6) and later, with the exception of CXCR4, (7) also in the other resolved structures of GPCRs (8). It lies perpendicular to the seven-transmembrane domain bundle of GPCRs and has been reported to move significantly upon receptor activation (9, 10). Therefore, helix 8 is an excellent candidate for the agonist-induced recognition of and interaction with cytosolic binding partners and has been studied extensively in several GPCRs. Studies have not only shown the involvement of helix 8 in G protein activation (11–15) but also identified hydrophobic residues in helix 8 as essential for the surface expression of the GPCRs investigated (11, 16, 17). In addition, it has been published only recently that mutation of positively charged residues in helix 8 of the thyrotropin releasing hormone receptor prevented GRK-mediated receptor phosphorylation (18).

As demonstrated by our research group, mutation of Tyr7.53 (Ballesteros/Weinstein nomenclature (19)) to an alanine in the NP*XX*Y sequence resulted in constitutive receptor phosphorylation and internalization (20). Homology modeling indicated that Tyr7.53 in the B_2R exerts an aromatic stacking interaction with the highly conserved phenylalanine Phe7.60 in helix 8. Thus, we speculated that the properties of mutant Tyr7.53 are

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: B_2R, bradykinin B_2 receptor; BK, bradykinin; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; P_{min}, minimal promoter; DSP, dithiobis[succinimidylpropionate]; IP, inositol phosphate; PAR2, protease-activated receptor-2; β_2ADR, β_2-adrenergic receptor; eYFP, enhanced yellow fluorescent protein; ANOVA, analysis of variance; LDS, lithium dodecyl sulfate.

caused by a modified interaction with GRKs and arrestins involving helix 8. Further studies using chimera of the B_2R with the non-sequestering bradykinin B_1 receptor also suggested that helix 8 might be involved directly or indirectly in the interaction of the B_2R with GRKs and β-arrestins (21).

Consequently, we investigated in the present study not only the function of helix 8 in the B_2R with regard to surface expression and G protein activation but also assessed its role in the desensitization process involving the interaction with GRKs and β-arrestins. Our results clearly demonstrate that helix 8 is involved in all of the before-mentioned receptor-dependent processes, however, to a different degree and with different requirements for the receptor microenvironment comprising helix 8.

EXPERIMENTAL PROCEDURES

Materials—Flp-In™ TREx-293 (HEK 293) cells, and Opti-MEM I serum-free medium were obtained from Invitrogen. [2,3-Prolyl-3,4-^{3}H]bradykinin (80 Ci/mmol) and myo-2-[^{3}H]inositol (22 Ci/mmol) were from PerkinElmer Life Sciences. Vectors harboring the genes of GRK2–6 were kindly provided by Dr. A. de Blasi (University of Rome), and a vector comprising the gene of the human β_2-adrenergic receptor was provided by Dr. M. J. Lohse (University of Würzburg). All other receptor sequences were obtained from the Missouri S&T cDNA Resource Center. Bradykinin was purchased from Bachem (Weil am Rhein, Germany). The B_2R antagonist JSM10292 was a generous gift from the Jerini AG (Berlin, Germany). Roche Applied Science delivered FuGENE HD, complete mini EDTA-free protease inhibitor tablets, and the rat monoclonal anti-hemagglutinin (HA)-peroxidase high affinity antibody (3F10). EcoTransfect was purchased from OzBiosciences (Marseille, France). EZview red anti-HA affinity gel was bought from Sigma. Cell culture reagents were obtained from PAA Laboratories (Cölbe, Germany). Monoclonal mouse anti-β-arrestin1 antibody and polyclonal rabbit anti-β-arrestin2 antibody were from BD Transduction Laboratories and Millipore (Billerica, MA), respectively. Rabbit polyclonal anti-GRK3 antibody (sc-563; C-14) was from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Two sets of siRNAs from Qiagen (Hilden, Germany) were used. The first set was predesigned HP-validated FlexiTube β-arrestin siRNAs: β-arrestin1 (catalog no. SI02776921, 5′-CGACGUUCUGCAAGGUCUATT-3′) and β-arrestin2 (catalog no. SI02776928, 5′-CGAACAAGAUGACCAGGUATT-3′). The second set was published siRNAs (22): β-arrestin1 (5′-AGCCUUCUGCGCGGAGAAUTT-3) and β-arrestin2 (5′-GGACCGCAAAGUGUUUGUGTT-3′). HiPerFect transfection reagent was acquired from Qiagen. The cross-linking agent dithiobis[succinimidylpropionate] (DSP) was purchased from Pierce.

Gene Mutagenesis and Expression of Constructs—Standard PCR techniques with appropriate primers were used to generate mutants of the B_2R, of GRK3, and the receptor chimeras. Coding sequences of the B_2R and the respective mutants started with the third encoded methionine (23). Receptor constructs, GRK3, β-arrestin1, and β-arrestin2 genes were all cloned into the HindIII and the XhoI sites of the pcDNA5/FRT/TO vector (Invitrogen). The receptor sequences were preceded at the N terminus by a single hemagglutinin tag (MGYPYDVPDYAGS) with the last two amino acids (Gly-Ser) of the tag being generated by the insertion of a BamHI site. For the N-terminal eYFP (enhanced yellow fluorescent protein) fusion constructs, the receptors were preceded by the signal sequence of the human frizzled receptor 4 followed by the coding sequence for the eYFP and the HA tag.

Cell Culture and Transfection—For stable expression of the constructs in HEK 293 cells we used the Flp-In system from Invitrogen as described previously (24). For transient transfection cells were seeded into 24 wells (80% confluency) and transfected using 0.2 μg of DNA and 0.4 μl of EcoTransfect. Expression was induced with 0.5 μg/ml tetracycline 5–6 h after transfection and 16 h before the respective assay. For β-arrestin knockdown, monolayers (50% confluent) in 24 wells were treated with 125 ng of siRNA/1.5 μl of HiPerFect 72 h before the experiment.

Equilibrium Binding Experiments at 4 °C—The dissociation constant (K_d) was determined with [^{3}H]BK as described previously (24). In brief, cells were incubated for 90 min on ice in incubation buffer (40 mM Pipes, 109 mM NaCl, 5 mM KCl, 0.1% glucose, 0.05% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing increasing concentrations of up to 30 nM [^{3}H]BK. Surface-bound [^{3}H]BK (>95% of totally bound radioactivity) was dissociated by a 10-min incubation with 0.2 ml of an ice-cold dissociation solution (0.5 M NaCl, 0.2 M acetic acid, pH 2.7), transferred to a scintillation vial, and counted in a β-counter after the addition of scintillation fluid.

Determination of Inositol Phosphate (IP) Release—Phosphatidylinositol hydrolysis was measured as previously described (24). In brief, confluent cell monolayers (24 wells) were incubated overnight in 0.5 ml of complete medium containing 1 μCi [^{3}H]inositol/ml. The cells were preincubated for 90 min on ice in incubation buffer supplemented with 50 mM LiCl with or without the addition of increasing concentrations of BK. Stimulation was started by placing the cells in a water bath at 37 °C. After 30 min IP accumulation was stopped by exchanging the medium for 1.5 ml of ice-cold 20 mM formic acid solution. After 1 h on ice and application of the supernatant to AG 1-X8 columns, total [^{3}H]IP was determined as described before (24).

[^{3}H]BK Internalization—Internalization of [^{3}H]BK was determined as described recently (24). Cells on multiwell plates (24/48 wells) were incubated with the indicated [^{3}H]BK concentration in incubation buffer for 60 min on ice to obtain equilibrium binding. [^{3}H]BK internalization was started by placing the plates in a water bath at 37 °C for the indicated times and stopped by putting them back on ice. Surface-bound [^{3}H]BK was dissociated by incubating the cells for 10 min with 0.2 M acetic acid, 0.5 M NaCl, pH 2.7. Thereafter, internalized [^{3}H]BK was determined by lysing the cell monolayer with NaOH (0.3 M). [^{3}H]BK in both samples was measured in a β-counter after the addition of scintillation fluid. Internalization was expressed as the amount of internalized [^{3}H]BK in the percentage of the combined amounts of internalized and surface-bound [^{3}H]BK.

For the determination of intrinsic receptor internalization properties (widely unaffected by high receptor expression and/or limited availability of endogenously expressed interac-

tion partners) we used [^{3}H]BK concentrations ≤1 nM (25). To detect endogenous factors that might limit the process (*e.g.* β-arrestins) we used 5 nM or higher concentrations as indicated.

Receptor Down-regulation—Monolayers (48-well) were incubated with or without 1 μM unlabeled BK in 0.5 ml of Opti-MEM I for the indicated times at 37 °C. Thereafter, plates were rinsed with ice-cold PBS and incubated on ice for 10 min with 0.2 ml of dissociation solution to remove all unlabeled extracellular ligand. The cells were washed again with ice-cold PBS, and specific binding was determined with 2 nM [^{3}H]BK at 4 °C by subtracting nonspecific binding (determined in the presence of 5 μM unlabeled BK) from total surface binding.

Co-immunoprecipitation—Confluent monolayers of cells stably expressing HA-tagged receptor constructs on 10-cm dishes were transfected with 5 μg of plasmid harboring the respective constructs and 10 μl of EcoTransfect 36 h before co-immunoprecipitation. Cells were washed twice with PBS, warmed up to 37 °C in a water bath, and subsequently stimulated with 1 μM BK for the indicated times in 4.8 ml of PBS. The stimulation was stopped by the addition of 0.2 ml of 25 mM cross-linking agent (DSP, dissolved in DMSO) to obtain 1 mM final concentration. After incubation for 20 min at room temperature, cells were rinsed 3 times with quenching solution (50 mM Tris-HCl, pH 7.4) and solubilized in 1 ml of lysis buffer (10 mM Tris-HCl, 25 mM KCl, 150 mM NaCl, 0.1% Triton X-100, pH 7.4) including protease inhibitors for 15 min at 4 °C with gentle agitation. After centrifugation at 17000 × *g* for 15 min at 4 °C, 20 μl of the supernatant was mixed with an equal amount of 2× LDS sample buffer containing 0.2 M DTT and incubated for 10 min at 95 °C; the residual supernatant was added to 20 μl of EZview red anti-HA affinity gel and incubated for 1 h under gentle agitation at 4 °C. Thereafter, the anti-HA matrix was washed 3 times with ice-cold lysis buffer, then 30 μl of 1× LDS sample buffer containing 0.1 M DTT was added, and the immunocomplexes were dissociated at 95 °C for 10 min. Samples were separated on a 4–12% SDS-polyacrylamide gel and transferred to a 0.45-μm nitrocellulose membrane, which was blocked for 1 h at room temperature with milk powder dissolved in TBST (Tris-buffered saline, pH 7.5, 0.1% Tween 20). Subsequently the membrane was incubated overnight at 4 °C with anti-β-arrestin2 antibody (1:1000) or for 1 h at room temperature with anti-β-arrestin1- or anti-GRK3 antibody, each diluted 1:1000 in blocking buffer. Thereafter, the membrane was washed with TBST and incubated with HRP-linked goat anti-rabbit IgG diluted 1:2000 in blocking solution. For the recognition of the primary mouse anti-β-arrestin1 antibody, the peroxidase-labeled anti-mouse true blot secondary antibody (1:2000) (eBioscience, San Diego, CA) was used. Antibody binding was detected using ECL solution according to the instructions of the manufacturer.

Immunoblotting—Confluent monolayers in 6-well trays were washed 3 times with ice-cold PBS and solubilized in 300 μl of lysis buffer and cleared by centrifugation as described above. Aliquots of the supernatant were mixed with equal amounts of 2× LDS sample buffer (0.2 M DTT) and incubated for 10 min at 95 °C, and proteins were electrophoresed, electroblotted, and detected as described above. For deglycosylation, 5 μl of 10× deglycosylation buffer (PBS, 20 mM EDTA, 1% SDS, 5% Triton X-100, 10% 2-mercaptoethanol) was added to 45 μl of clear cell lysate and denatured at 80 °C for 10 min. After the addition of 1 unit of *N*-glycosidase F, the samples were incubated for 2 h at 37 °C without agitation and mixed with 1× LDS sample buffer (0.1 M DTT). HA-tagged receptors were visualized after 1 h of blocking in 5% milk powder at room temperature followed by 1 h of incubation with a monoclonal HRP-linked anti-HA high affinity antibody (1:2000) diluted in fresh blocking buffer.

Surface Biotinylation—Cells stably expressing receptor constructs were plated on 6 well and incubated for 3 h at 37 °C in Opti-MEM I containing ligand or vehicle. Subsequently, cells were washed with ice-cold PBS and incubated on ice for 30 min with a solution of 0.3 mg/ml thiol-cleavable sulfo-NHS-SS-biotin (Pierce) dissolved in PBS. After cell lysis, biotinylated receptors were precipitated and run on a SDS-PAGE as described above. Dissociation of precipitated receptors was performed in DTT-free Laemmli buffer to avoid cleavage of the biotin linker. After blotting, the receptors were detected with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

ERK1/2 Phosphorylation—Cells stably expressing GRKs 2–6 were plated on 24 wells and transfected with the B_2Rwt_H. After 12 h the culture medium was changed for DMEM containing reduced FCS (0.5%) and 0.5 μg/ml tetracycline to induce gene expression. Another 36 h later the cells were washed with ice-cold PBS and incubated on ice in 0.15-ml incubation buffer containing 1 μM BK. After 30 min, the plates were put in a 37 °C warm water bath for the indicated times. Subsequently the cells were solubilized in 0.2 ml of lysis buffer, and proteins were separated by SDS-PAGE as described above. After electroblotting and blocking with 5% milk powder in TBST, phospho-ERK1/2 and total-ERK1/2 were detected with phospho-p44/42 MAPK (E10) and a p44/42 MAP kinase (3A7) mouse monoclonal antibody (Cell Signaling, Frankfurt, Germany), respectively.

Epifluorescence Microscopy—Cells stably expressing eYFP-receptor fusion proteins were seeded on glass coverslips. The cell culture medium was changed to Opti-MEM I medium before incubation with 1 μM BK or 1 μM JSM10292. Images were taken with a Olympus IX70 fluorescence microscope.

Data Analysis—All experiments were performed at least three times in duplicate or triplicate, and results are given as the mean ± S.E. unless otherwise indicated. Data analysis was carried out using GraphPad Prism for Macintosh, Version 4.0c (GraphPad Software, Inc.).

RESULTS

Helix 8 Requirement for High B_2R Cell Surface Expression—To investigate the importance of helix 8 for the function of the B_2R, we generated several constructs with increased truncations of the C terminus up to the center of helix 8 (Fig. 1*A*). Moreover, to disturb the structure of helix 8 by a single point mutation, leaving the B_2R C terminus otherwise unchanged, we mutated Lys-315, located in the center of helix 8, to proline (construct K315P), a known helix breaker (Fig. 1*A*). As a control that altered properties observed in K315P are not caused by Lys-315 being an important residue *per se*, *e.g.* as a target for

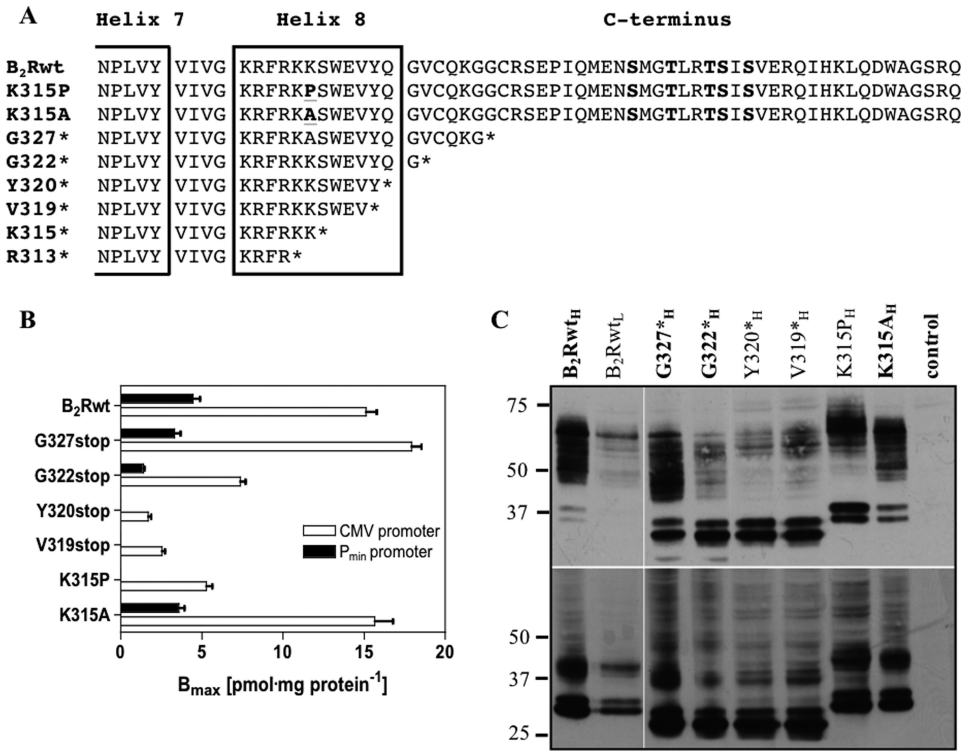

FIGURE 1. **B_2R constructs and their expression levels in HEK 293 cells.** *A*, shown is a schematic representation of the C-terminal amino acid sequences of the human B_2Rwt and the mutant receptor constructs. Alignment starts after the conserved NP*XX*Y motif after transmembrane helix 7. The predicted helix 8 is framed, and the point mutations (K315P, K315A) are *bold* and *underlined*; the C-terminal serine/threonine phosphorylation sites are in *bold*. *B*, maximal numbers of binding sites were determined with 30 nM [^{3}H]BK. *Open columns*, expression of constructs under the control of the CMV promoter; *filled columns*, expression of constructs under the weaker P_{min} promoter. *C*, cells stably expressing HA-tagged B_2Rwt and mutants (inferior H and L for high and low expression) were lysed and separated by SDS-PAGE followed by Western blotting and detection with a monoclonal HA-antibody (*upper panel*). Aliquots of the same lysates were incubated with 1 units of *N*-glycosidase F as described under "Experimental Procedures" but otherwise treated identically (*lower panel*). 5 μg protein was applied for constructs marked in *bold*, and 15 μg of protein was applied for the remaining constructs. *Numbers on the left* represent relative molecular masses (in kDa).

ubiquitination or for the overall receptor structure, mutant K315A was generated as well.

Employing the Flp-In system (Invitrogen), all N-terminally HA-tagged receptor constructs became stably integrated at an identical unique locus in the genome of the HEK 293 host cell line under the control of the strong cytomegalovirus (CMV) promoter. Construct G327* (G327stop), displayed with about 18 pmol·mg^{-1} protein^{-1} similar maximal surface receptor numbers (B_{max}) as the wild-type B_2R (B_2Rwt) (Fig. 1*B*, Table 1). However, truncations of the C terminus proximal to Cys-324 resulted in progressively reduced surface expression down to 2–3 pmol·mg^{-1} protein^{-1}. Lower surface receptor numbers were also observed for mutant K315P (5.3 pmol·mg^{-1} protein) but not for mutant K315A (~16 pmol·mg^{-1} protein) (Fig. 1*B*; Table 1). These results demonstrate that the distal C terminus of the B_2R is not required for high surface expression but that an intact helix 8 contributes to it as being reported for other GPCRs (11, 16, 17, 26). To be able to directly compare the properties of the different constructs without having to consider the potential influence of the highly different surface expression levels, we also generated cell lines, where the normally highly expressed constructs (B_2Rwt, G327*, G322*, K315A) were expressed at distinctly lower levels under the control of the weaker P_{min} promoter (Fig. 1*B*; Table 1, constructs were denoted with inferior L or H for low and high expression, respectively). These distinctly lower surface expression levels were similar to those determined for mutants, which exhibit low levels despite being under the control of the strong CMV promoter, *i.e.* constructs Y320*, V319*, and K315P.

Immunoblot analysis of the expressed constructs using the N-terminal HA tag revealed several bands between 45 and 75 kDa as well as prominent double bands below 40 kDa (Fig. 1*C*, *upper panel*). All truncations made proximal to Cys-324 (G322*, Y320*, Y319*) displayed a weaker pattern of bands between 40 and 75 kDa but very strong double bands. Pretreatment of protein lysates with *N*-glycosidase F resulted for all samples in the appearance of one additional major lower band and more intense double bands (Fig. 1*C*, *lower panel*), demonstrating that the different bands reflect different glycosylation states and that also the double bands represent intact receptor protein and not degradation products. These results point to a role of helix 8 in the B_2R in stabilizing a proper conformation that allows for full glycosylation and efficient trafficking to the plasma membrane.

Reduced G Protein Activation of Helix 8 Mutants—To determine the participation of helix 8 in G protein activation, we measured the accumulation of total IPs in the respective cell lines after stimulation with increasing concentrations of BK for 30 min at 37 °C. Mutant G327*, lacking the whole C terminus, exhibited an almost 30-fold lower EC_{50} value than B_2Rwt and also elevated levels (~2–3-fold as compared with B_2Rwt) of basal IP accumulation (Fig. 2; Table 1).

TABLE 1

[^{3}H]BK binding data and BK-induced IP accumulation

Number of experiments are given in parentheses. ND, not determined. Significance was determined by one-way ANOVA using Dunnett's multiple comparison test. *, position of the stop codon in each truncated receptor.

Receptor construct[a]	[^{3}H]BK binding		IP accumulation		
	Bmax[b]	K_d (4°)	Basal[c]	Maximal effect[c]	EC_{50}[d]
	pmol/mg protein	*nM*			*nM*
B_2Rwt_H	15.1 ± 0.7 (6)	2.90 ± 0.32 (19)	2.02 ± 0.13 (10)	12.55 ± 1.00	0.83 ± 0.22 (6)
B_2Rwt_L	4.5 ± 0.4 (6)				
$K315P_H$	5.3 ± 0.4 (6)	6.61 ± 0.58 (5)	1.78 ± 0.16 (4)	8.28 ± 0.02	5.96 ± 0.73 (4)[e]
$K315A_H$	15.6 ± 1.1 (3)	7.14 ± 1.35 (4)	2.28 ± 0.17 (3)	9.66 ± 1.52	0.28 ± 0.07 (3)
$K315A_L$	3.6 ± 0.4 (3)				
$G327^*_H$	17.9 ± 0.7 (3)	4.83 ± 0.84 (9)	8.81 ± 1.60 (4)	13.58 ± 1.22	0.03 ± 0.01 (5)
$G327^*_L$	3.3 ± 0.4 (3)				
$G322^*_H$	7.4 ± 0.3 (3)	3.42 ± 0.73 (5)	ND	ND	ND
$G322^*_L$	1.4 ± 0.1 (3)				
$Y320^*_H$	1.7 ± 0.2 (6)	2.63 ± 0.52 (9)	1.61 ± 0.31 (6)	11.08 ± 1.48	2.27 ± 0.95 (3)
$V319^*_H$	2.6 ± 0.2 (6)	2.99 ± 0.70 (7)	1.64 ± 0.08 (7)	7.75 ± 0.89	6.01 ± 1.26 (4)[e]
$K315^*_H$	3.2 ± 0.4 (3)	3.79 ± 0.22 (3)	1.67 ± 0.16 (3)	9.39 ± 1.08	10.63 ± 1.54 (4)[e]
$R313^*_H$	2.7 ± 0.2 (3)	6.51 ± 1.13 (3)	1.78 ± 0.09 (3)	3.98 ± 0.13	ND

[a] Receptor constructs were isogenically and stably expressed under the control of the strong CMV promoter or the weaker P_{min} promoter. Expression levels are indicated with inferior H for high and L for low.
[b] Estimated with 30 nM [^{3}H]BK on ice.
[c] Total IP accumulation after 30 min of incubation in buffer with inhibitors and 50 mM LiCl at 37 °C without (basal) and with (maximal effect) 1 μM BK, expressed as the -fold increase of initial total IP content ($t = 0$ min).
[d] Calculated from incubations in duplicates with 10^{-12}–10^{-5} M BK for 30 min at 37 °C in the presence of 50 mM LiCl.
[e] $p < 0.01$.

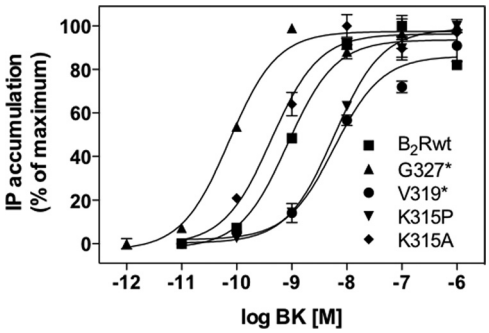

FIGURE 2. **Dose-dependent IP release.** HEK 293 cells stably expressing B_2Rwt or the mutants were stimulated with the indicated concentrations of BK for 30 min at 37 °C. Total inositol phosphate accumulations are shown as representative sigmoidal dose-response curves, normalized for maximal response (100%).

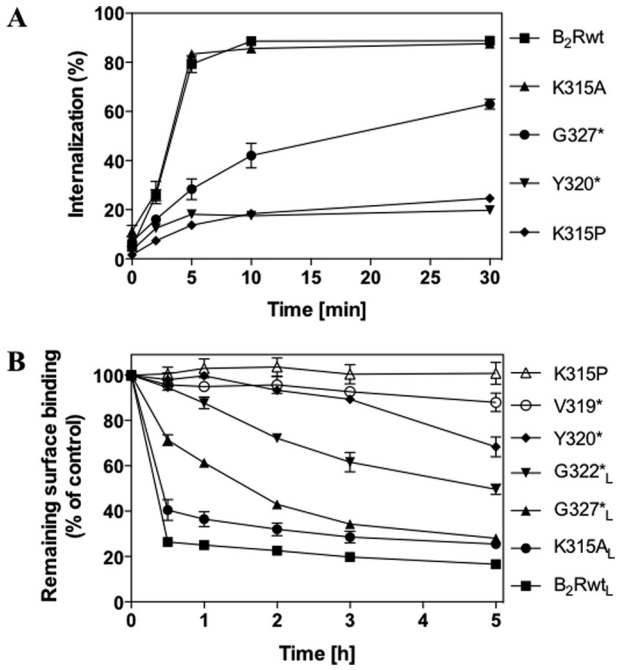

FIGURE 3. **[^{3}H]BK internalization and long term down-regulation.** *A*, HEK 293 cells stably expressing B_2Rwt and the shown constructs were preincubated with 1 nM [^{3}H]BK for 60 min on ice and subsequently warmed up to 37 °C to start internalization. At the indicated times surface-bound and internalized [^{3}H]BK were determined as described under "Experimental Procedures." Internalization is given as a percentage of total bound [^{3}H]BK (surface plus internalized [^{3}H]BK). *B*, HEK 293 cells expressing B_2Rwt or mutants were incubated in the presence of 1 μM BK at 37 °C for the indicated times. Subsequently cells were treated with NaCl/acetic acid solution to remove all unlabeled agonist. Remaining surface binding was determined with 2 nM [^{3}H]BK at 4 °C and is given in a percentage of control (not treated with BK). Constructs denoted with an inferior L (for low expression) were expressed under the control of the P_{min} promoter.

In contrast, all mutants with a defective helix 8 (including K315P) displayed ~3–8-fold lower G protein-coupling efficiencies, whereas the maximal responses were comparable for all constructs (Fig. 2; Table 1). Mutant K315A behaved very much like B_2Rwt, demonstrating that a positive charge in this position is not required for G protein activation. Together, all mutants showed robust phosphatidylinositol hydrolysis, indicating that the overall receptor structure is largely unaffected by the mutations. But helix 8 must directly or indirectly participate in the G protein activation of the B_2R as the EC_{50} values of the helix 8-defective mutants clearly differed from that of B_2Rwt.

Requirement of an Intact Helix 8 for B_2R-mediated [^{3}H]BK Internalization—After stimulation with BK, the B_2R becomes rapidly sequestered to compartments within the cell. To investigate the role of helix 8 for receptor sequestration, we examined the capability of the receptor constructs to internalize [^{3}H]BK. As a consequence of the lacking C terminus, construct G327* displayed strongly reduced internalization of ^{3}[H]BK as compared with the B_2Rwt (Fig. 3*A*). Mutants with a disturbed helix 8 were not capable of internalizing [^{3}H]BK at all, whereas construct K315A behaved like B_2Rwt. These results show that lack of the receptor C terminus with the phosphorylation sites only reduces rapid receptor sequestration, whereas a defective helix 8 prevents it almost completely as best demonstrated with K315P, a construct still comprising the complete C terminus with all phosphorylation sites (Fig. 3*A*).

Requirement of an Intact Helix 8 for Sequestration upon Long Term Stimulation—We next investigated how the mutations affect the outcome of long term stimulation by treating the cells with a saturating concentration of BK (1 μM) for up to 5 h at 37 °C. Remaining surface binding was determined with 2 nM ^{3}[H]BK at 4 °C after removal of all unlabeled BK with NaCl/acetic acid solution. As receptor sequestration is considerably slowed down *per se* by high expression levels of constructs (25), we used the stable cell lines expressing receptor genes under the control of the weaker P_{min} promoter when available. Cell surface binding was reduced by more than 80% after 30 min for the B_2Rwt_L and remained at that level for at least 5 h (Fig. 3*B*). Even though receptor numbers decreased strikingly slower for G327*$_L$ than for B_2Rwt_L, they reached a similarly low level after 5 h of BK treatment. The degree of receptor sequestration was progressively reduced with shortening the C terminus and was completely prevented in mutation V319*, indicating that an intact helix 8 is crucial for the sequestration of the B_2R. This notion was strengthened by the result that mutant K315P also showed no reduction of surface binding within the 5-h time frame of stimulation with BK, whereas K315A$_L$ behaved like B_2Rwt_L.

Epifluorescence Microscopy with N-terminally eYFP-tagged Constructs—To visualize the localization of selected receptor variants without affecting the function of the C terminus, we generated fusion proteins with eYFP joined to their N terminus. To allow for proper extracellular expression, the eYFP sequence was preceded by the signal sequence of the human frizzled 4 receptor as otherwise the fusion proteins were trapped inside the cell. The integrity of the fusion constructs was confirmed by Western blot analysis (not shown). Epifluorescence microscopy revealed a strongly dispersed intracellular localization of eYFP-V319* (not shown here) and of eYFP-K315P (Fig. 4*E*), whereas eYFP-B_2Rwt (Fig. 4*A*) and eYFP-G327* (Fig. 4*C*) were almost exclusively located at the plasma membrane. In these latter two cell lines, a strong redistribution of the receptors from the plasma membrane to intracellular vesicles after BK treatment became obvious, which proceeded more rapidly for B_2Rwt (shown after 30 min, Fig. 4*B*) than for G327* (shown after 120 min, Fig. 4*D*), in accordance with the sequestration data given in Fig. 3*B*. The fusion constructs of V319* and K315P did not display any recognizable redistribution even upon prolonged stimulation (up to 5 h; not shown here). However, when these two constructs were treated for 3 h with the small molecule B_2R antagonist JSM10292 (27),[3] they were found predominantly at the cell surface (shown here only for eYFP-K315P (Fig. 4*F*).

These results were confirmed by cell surface biotinylation followed by immunoprecipitation. Whereas the number of biotinylated cell surface receptors was strongly reduced for B_2Rwt and mutant G327* after a 3-h incubation with BK, no change was observed for mutant K315P (Fig. 4*H*). However, for the latter mutant a significant increase was observed upon incubation with JSM10292 for 3 h (Fig. 4, *G* and *H*). A 16-h incubation of mutant K315P with this antagonist resulted even in a change to an immunoblot pattern that resembled that of the B_2Rwt (Fig. 4*I*). These results suggest that the said B_2R antagonist can correct the trafficking to the plasma membrane, hampered by the disturbed helix 8.

Together these results confirm that rapid receptor internalization is dependent on the presence of the C terminus. Yet slower sequestration can occur in a C terminus-independent way as well. For both processes, however, an intact helix 8 is crucial.

C-terminal-independent B_2R Internalization Mediated by β-Arrestin2—The ubiquitously expressed β-arrestins1 and -2 have been shown to target GPCRs to clathrin-coated pits through their interaction with clathrin and the adapter protein AP-2, thereby promoting receptor internalization (28, 29). Furthermore, Simaan *et al.* (30) have reported that in COS7 cells β-arrestin2 mediates B_2R internalization. To investigate whether C-terminal-dependent and -independent receptor endocytosis have different requirements for β-arrestins, we used siRNA technology to reduce the endogenous levels of β-arrestin1 and -2 in HEK 293 cells (Fig. 5*B*).

Single knockdown of β-arrestin1 had no significant impact on B_2Rwt internalization (Fig. 5*A*). Knockdown of β-arrestin2 reduced receptor endocytosis by 40%, which could be further significantly decreased by an additional β-arrestin1 knockdown, indicating a partly compensatory effect of β-arrestin1 in the case of diminished β-arrestin2 levels (Fig. 5*A*). The latter effect did not turn significant with a second set of β-arrestin siRNAs (supplemental Fig. S2*A*). Comparing the two different sets of siRNAs for their ability to reduce overexpressed β-arrestin1 and -2 levels, the first set proved to be much more efficient than the second (supplemental Fig. S2*B*). This might explain why the additional effect of β-arrestin1 was not observed with the second siRNA set, as it can apparently be observed only with a highly efficient β-arrestin2 siRNA.

Receptor internalization of stably transfected G327* in HEK 293 cells was monitored using 1 nM [^{3}H]BK for 15 min, as higher concentrations made receptor endocytosis hardly detectable for that mutant. Only knockdown of β-arrestin2, but not of β-arrestin1, significantly reduced internalization of G327* (Fig. 5*A*). Simultaneous knockdown of both β-arrestin isoforms could not further lower receptor internalization, indicating a subtype-specific role of β-arrestin2 for the C terminus-independent internalization.

We next examined [^{3}H]BK internalization of transiently transfected B_2Rwt in HEK 293 cells, which stably overexpressed β-arrestin1 or β-arrestin2 (Fig. 5*D*). Only minor enhancing effects of β-arrestin2 but not of β-arrestin1 overexpression on [^{3}H]BK internalization of B_2Rwt were observed (Fig. 5*C*). However, under certain circumstances β-arrestin1 can also play a role in this process as β-arrestin1 overexpression was able to rescue internalization that had been reduced by siRNA knockdown of endogenous β-arrestin2 (Fig. 5*C*). In contrast, overexpression of both β-arrestin subtypes strongly enhanced ligand-induced internalization of G327*, with β-arrestin2 exhibiting a significantly larger impact (Fig. 5*C*).

To directly assess the interaction of β-arrestins with receptor constructs, we performed co-immunoprecipitation with the chemical cross-linker DSP using cells stably overexpressing

[3] A. Faussner, S. Schüssler, J. Feierler, M. Bermudez, J. Pfeifer, K. Schnatbaum, T. Tradler, M. Jochum, G. Wolber, and C. Gibson, submitted for publication.

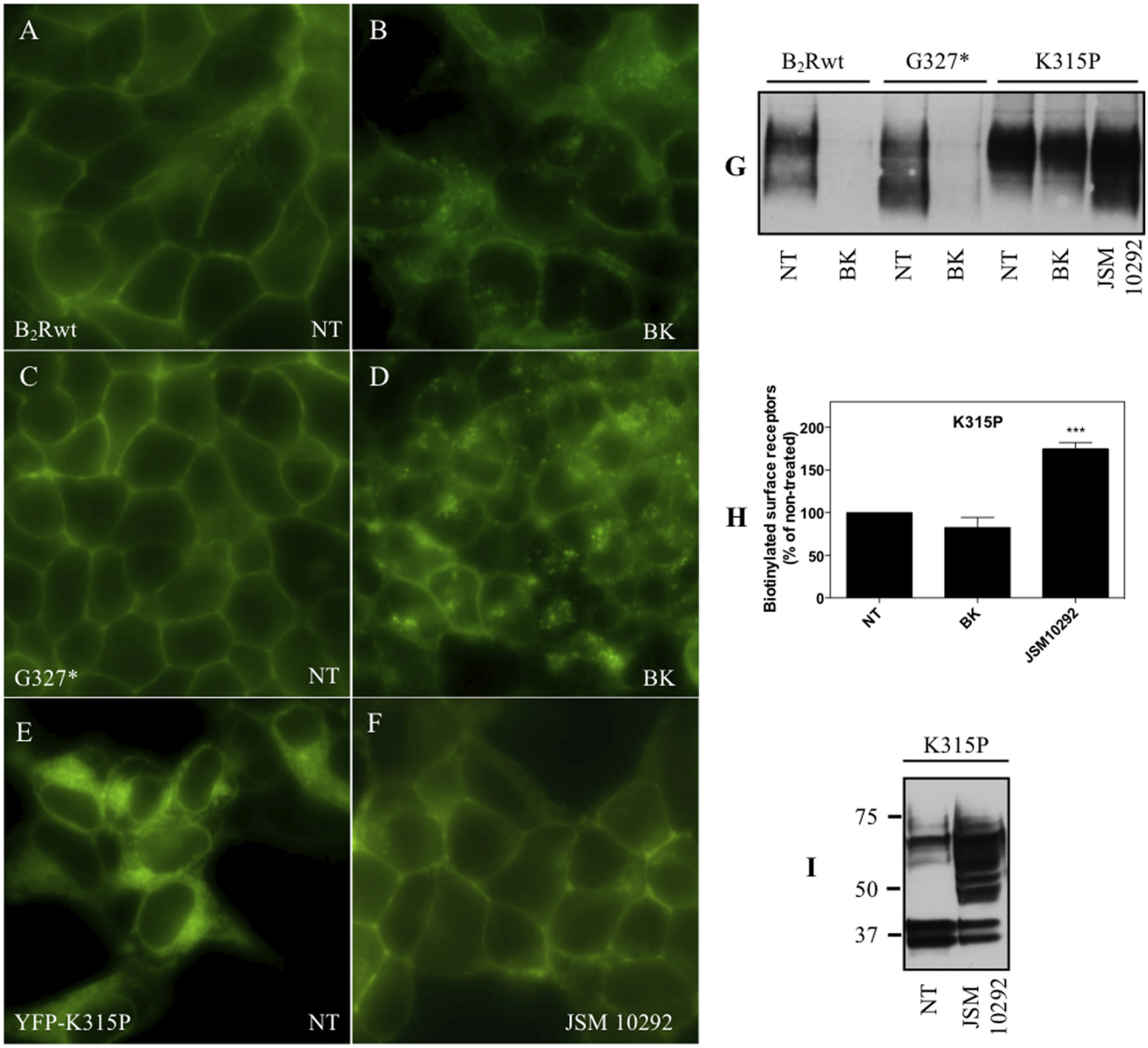

FIGURE 4. **Cellular distribution of B_2Rwt, G327*, and K315P.** *A–F*, HEK 293 cells stably expressing eYFP-B_2Rwt or eYFP-G327* were incubated at 37 °C in the absence (*NT*) or presence of 1 μM BK for 30 min (eYFP-B2Rwt) or 120 min (eYFP-G327*). Mutant eYFP-K315P was treated 3 h with the small molecule antagonist JSM10292. The eYFP fusion constructs were visualized by fluorescence microscopy. *G*, surface expression levels of receptor proteins were determined with a membrane-impermeable biotinylated linker before (*NT*) and after 3 h of treatment of BK or JSM10292 as described under "Experimental Procedures." *H*, shown is quantification of biotin-reactive bands using Image J software. Receptor density is given as the mean ± S.E. of four different experiments in percentage of the non-treated control. Significance was determined by one-way ANOVA using Bonferroni's multiple comparison test. ***, $p < 0.001$. *I*, shown is a representative immunoblot of K315P (~15 μg protein) treated without or with JSM10292 for 16 h

B_2Rwt_H or $G327^*_H$. Each cell line was transiently transfected with either β-arrestin1 or β-arrestin2. Both arrestins could be co-immunoprecipitated with the B_2Rwt after 5 min of stimulation (Fig. 5*E*). Mutant G327* still bound β-arrestin2, but not β-arrestin1, upon BK treatment, albeit obviously to a lower extent than the B_2Rwt.

These results suggest that internalization of G327* and B_2Rwt is largely mediated by the action of β-arrestin2, although β-arrestin1 is able to functionally compensate in part for β-arrestin2 under certain circumstances (*e.g.* such as overexpression of β-arrestin1 or after β-arrestin2 knockdown). Furthermore, the C terminus of the B_2R is less important for the interaction with β-arrestin2 than for coupling of β-arrestin1.

Regulation of B_2R-induced ERK1/2 Activation by GRK2 and GRK3—GRKs phosphorylate almost exclusively agonist-activated receptors, and consequently there must be molecular determinants of the receptor that signal the agonist-bound status to the GRKs. We hypothesized that helix 8 might function as such a signaling determinant. Overexpression of the ubiquitously expressed GRKs 2–6 produced a different phosphorylation pattern of the B_2R after stimulation (31); however, so far it was not investigated whether this also results in different signaling outcomes. Therefore, we first determined in our study how overexpression of GRKs 2–6 in HEK 293 cells (supplemental Fig. S1*A*) affects ERK1/2 activation by the B_2R. Of all here-examined GRKs, only overexpression of GRK2 and -3 sig-

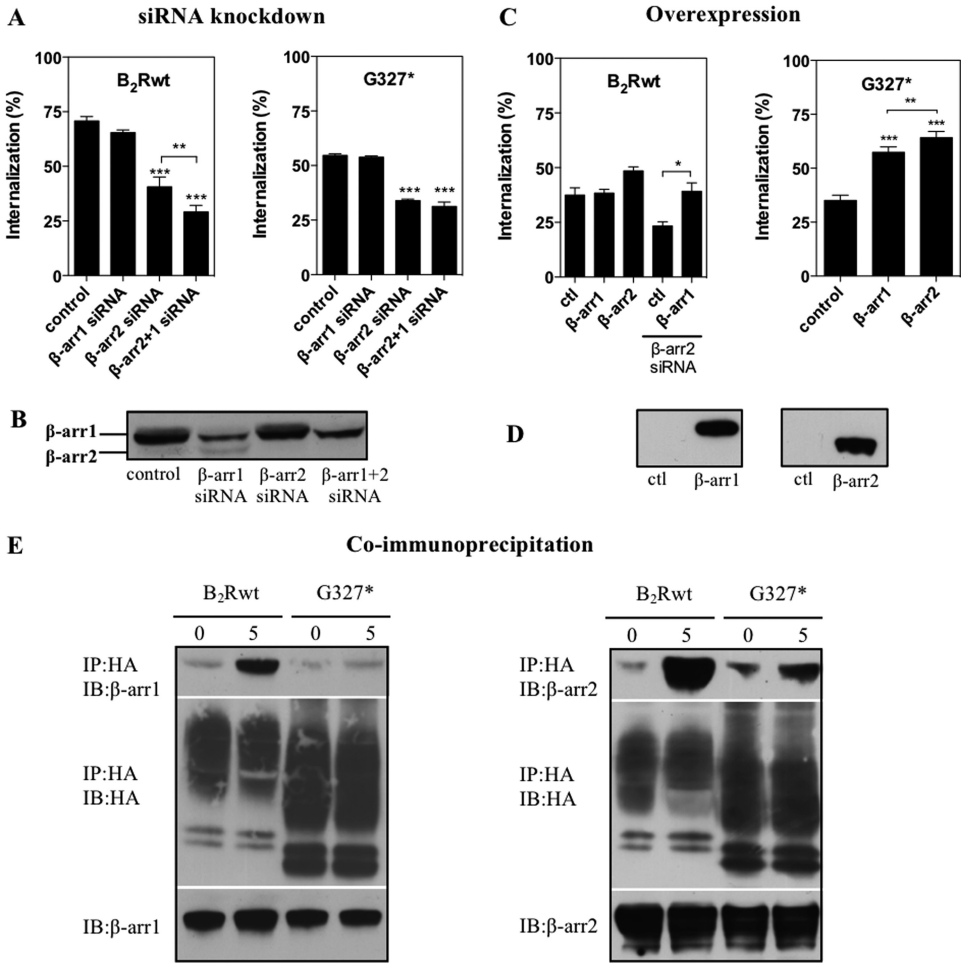

FIGURE 5. **Subtype-specific role of β-arrestin2 for the internalization of B_2Rwt and G327*.** *A*, HEK 293 cells stably expressing B_2Rwt or G327* were transfected with the indicated siRNAs. 72 h later internalization was determined with 5 nM [^{3}H]BK (*B_2Rwt*) or 1 nM [^{3}H]BK (*G327**) after 5 min (*B_2Rwt*) or 15 min (*B_2Rwt*) as described under "Experimental Procedures." *B*, shown is a representative immunoblot of endogenous β-arrestin levels in HEK 293 cells silenced with the indicated siRNAs and probed with a monoclonal β-arrestin1 antibody recognizing both subtypes. *C*, HEK 293 cells stably expressing β-arrestin1 or β-arrestin2 were transfected with B_2Rwt or G327*, and internalization was determined after 48 h with 5 nM [^{3}H]BK (B_2Rwt) or 1 nM [^{3}H]BK (*G327**) after 5 min (*B_2Rwt*) or 15 min (*G327**) at 37 °C. Where indicated, cells were pretreated with β-arrestin2 siRNA. *D*, shown is a representative immunoblot of cell lysates using ~2 μg of protein of cells overexpressing β-arrestin1 and -2. Statistical analysis was done with one-way ANOVA using Bonferroni's multiple comparison test. ***, $p < 0.001$, **, $p < 0.01$; *, $p < 0.05$. *ctl*, control. *E*, HEK 293 cells stably expressing HA-tagged B_2Rwt_H or G327*$_H$ were transiently transfected with β-arrestin1 (*left*) or β-arrestin2 (*right*) and stimulated (or not) with 1 μM BK for 5 min at 37 °C. After cross-linking with DSP and cell lysis, the protein complexes were precipitated with anti-HA-matrix. Precipitates were separated by reducing SDS-PAGE, transferred onto nitrocellulose membranes, and probed for β-arrestin1 (*left*) or β-arrestin2 (*right*) (*top panels*). Blots were stripped and re-probed to confirm receptor expression (*middle panels*), and lysates were tested for β-arrestin expression (*bottom panels*). *IP*, immunoprecipitation; *IB*, immunoblot.

nificantly reduced the ERK1/2 signal during the determination period of 30 min (supplemental Fig. S1, *B* and *C*).

Promotion of Phosphorylation-independent Internalization by GRK3—As GRK3 displayed the strongest effect, we chose this kinase to investigate the importance of helix 8 for the interaction of the B_2R with GRKs. Stimulation with BK resulted after 2 min in a stable interaction of GRK3 with the B_2Rwt as well as with G327*, which lasted for at least 30 min (Fig. 6*A*). Hence, GRK3 interaction can occur independently of the C terminus, its substrate, raising the question of whether there are other functions of GRKs besides the mere phosphorylation of the receptor C terminus. Overexpression of GRK2 or -3 resulted in minor, but significant increases in the internalization of the B_2Rwt (not shown). However, also the internalization of G327* (lacking the C terminus with all the phosphorylation sites) was significantly increased by overexpression of GRK3 (Fig. 6*B*).

It has been shown that GRKs contain consensus clathrin-binding motifs through which they are able to promote receptor internalization (32, 33). To discriminate whether clathrin binding and/or kinase activity promotes internalization of G327*, we generated a GRK3 variant with a group mutation of the clathrin binding motif to alanines (^{498}LLDCD^{502} → ^{498}AAAAA^{502}; GRK3-5A) in addition to a kinase activity-deficient mutant GRK3-K220R (Fig. 6*C*). Overexpression of GRK3-5A or GRK3-K220R did not increase significantly the internalization of G327* (Fig. 6*B*). Thus, the capability of GRK3 to promote B_2R endocytosis independently of the C-terminal phosphorylation sites still requires its kinase activity and the simultaneous presence of the clathrin binding motif.

Requirement of the Receptor C Terminus for Rescue of K315P Internalization by GRK3 and β-Arrestin2—Next, we investigated whether overexpression of GRK3 and β-arrestin1 or -2

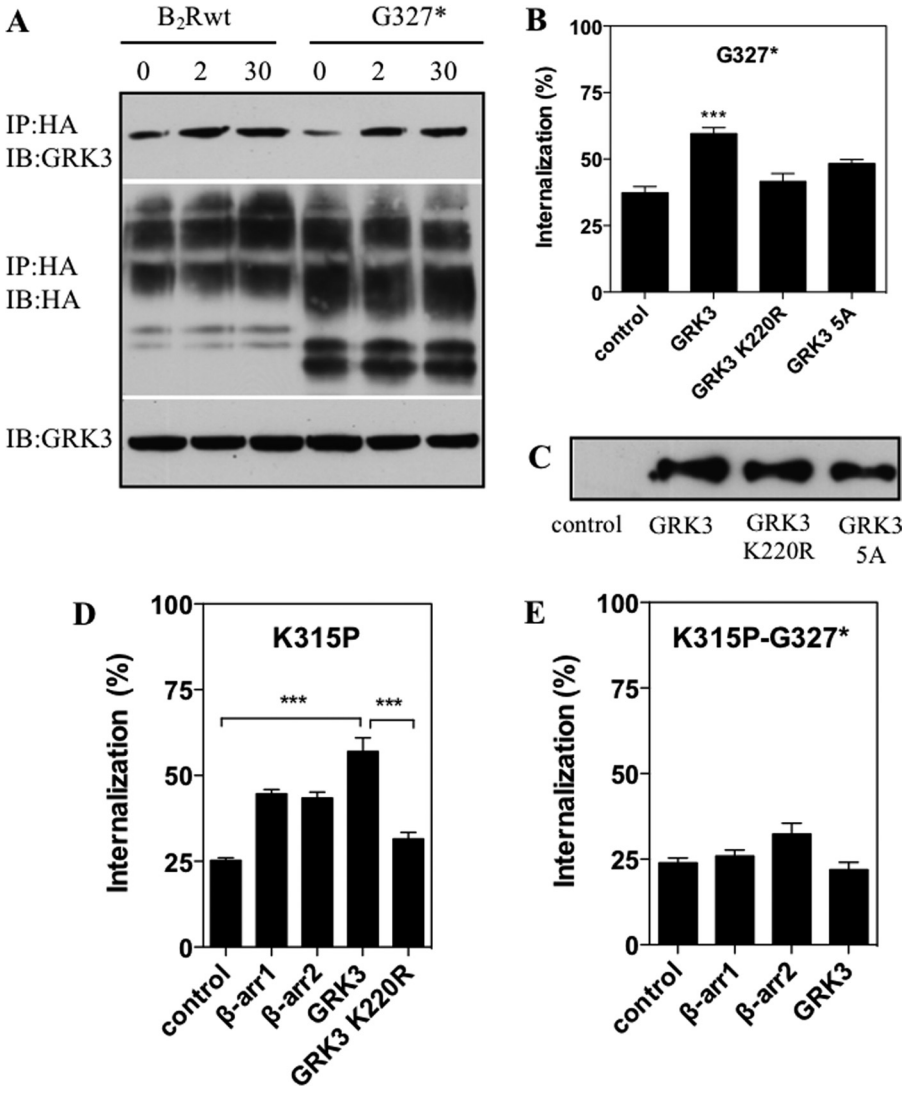

FIGURE 6. **C terminus-independent GRK interaction and rescue of K315P internalization.** *A*, HEK 293 cells stably expressing HA-tagged B_2Rwt or G327* were transiently transfected with GRK3 and stimulated (or not) with 1 μM BK for 2 or 30 min at 37 °C. After cross-linking with DSP and cell lysis, the protein complexes were precipitated with anti-HA matrix. Precipitates were separated by reducing SDS-PAGE, transferred onto nitrocellulose membranes, and probed for GRK3 (*top panel*). Blots were stripped and re-probed to confirm receptor expression (*middle panel*), and lysates were probed for GRK expression (*bottom panel*). *IP*, immunoprecipitation; *IB*, immunoblot. *B*, cells stably expressing GRK3wt, GRK3-5A, or GRK3-K220R were transiently transfected with G327*, and internalization was monitored with 1 nM [^{3}H]BK for 15 min at 37 °C. Significance was determined by one-way ANOVA using Bonferroni's multiple comparison test. ***, $p < 0.001$. *C*, a representative immunoblot of cell lysates using ~2 μg protein of cells (over)expressing GRK3wt, GRK3-K220R, and GRK3-5A is shown. *D* and *E*, HEK 293 cells stably (over)expressing the indicated β-arrestin subtypes or GRK3 constructs were transiently transfected with either K315P or K315P-G327*. Internalization was monitored for 15 min at 37 °C with 1 nM ^{3}H. Significance was determined by one-way ANOVA using Bonferroni's multiple comparison test. ***, $p < 0.001$

would be able to rescue the internalization of mutant K315P, which had a disturbed helix 8 but otherwise an intact C terminus. Overexpression of GRK3 resulted in a strong increase of ligand-induced internalization of K315P more than 2-fold, whereas that of the kinase-dead mutant GRK3-K220R did not, indicating the requirement of phosphorylation for the internalization process (Fig. 6*D*). Internalization of K315P could also be rescued by β-arrestin overexpression independently of the subtype, albeit to a smaller extent as compared with GRK3 (Fig. 6*D*). In contrast, in HEK 293 cells expressing the receptor mutant K315P-G327* (which combined the defective helix 8 with the lack of the distal C terminus) neither overexpression of β-arrestins nor GRK3 could significantly re-induce internalization (Fig. 6*E*), suggesting that the rescue by GRK3 or β-arrestins is strongly dependent on the presence of the intact C terminus or an intact helix 8.

Requirement of a Specific Microenvironment for Proper Function of Helix 8—Finally, to test whether C-terminal sequences and/or helices 8 of different GPCRs of family A are functionally transferable to the B_2R, we generated various chimeras of the B_2R with the C termini/helices 8 of the protease-activated receptor-2, the β_2-adrenergic receptor, the angiotensin II type 1 receptor, and the B_1 bradykinin receptor. Except B_1 bradykinin receptor, all these receptors become phosphorylated and internalized after agonist stimulation (34–37).

All chimeric constructs (Fig. 7*A*) were robustly expressed on the cell surface (~5–10 pmol·mg^{-1}protein^{-1}) as determined by radioligand binding assays with [^{3}H]BK. The chimeras with

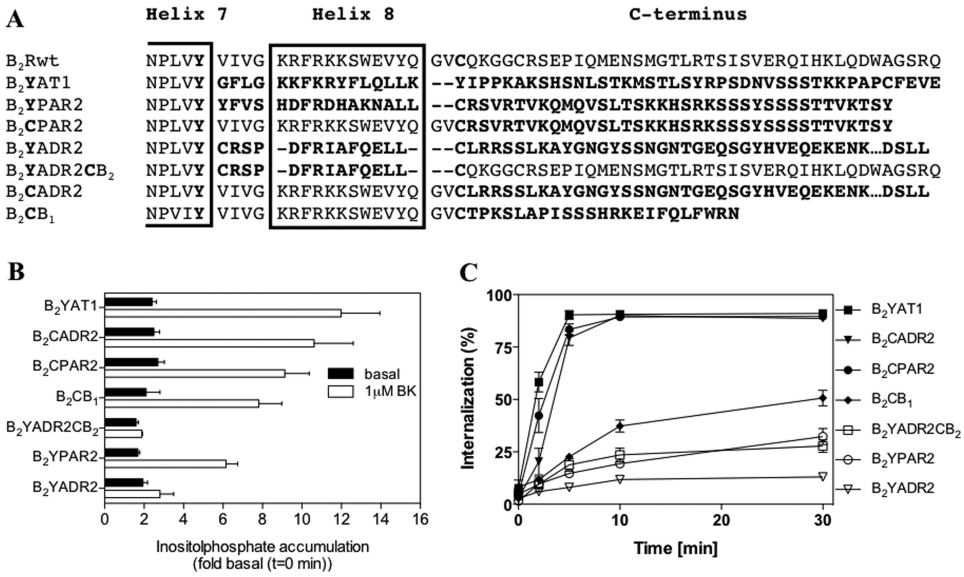

FIGURE 7. **Internalization and signaling of chimeric constructs.** *A*, alignment of the C-terminal residues of the human B_2Rwt swapped with sequences of various other GPCRs (marked in *bold*). Sequences were exchanged directly after the NP*XX*Y motif of transmembrane helix 7 or after the proximal Cys-324, respectively. The predicted helix 8 is framed. *B*, inositol phosphate release of B_2Rwt and chimeras, stably expressed in HEK 293 cells, was determined with 1 μM BK as described under "Experimental Procedures." The results are presented as -fold increase over the IP content of identically treated control cells that had remained on ice. *C*, internalization of B_2Rwt and chimeras stably expressed in HEK 293 cells was determined as described under "Experimental Procedures."

transfer of the C termini distal to helix 8 (B_2**C**PAR2, B_2**C**ADR2, B_2**C**B_1) showed strong phosphatidylinositol hydrolysis (Fig. 7*B*). For the other chimeras with transfer of helix 8 either with or without the distal C terminus phosphatidylinositol hydrolysis apparently depended on the source of helix 8. When helix 8 was derived from another G protein $G_{q/11}$-coupled GPCR (protease-activated receptor-2, angiotensin II type 1 receptor), the chimeras displayed robust signaling, whereas there was no signaling when helix 8 was taken from the β_2ADR (constructs B_2**Y**ADR2, B_2**Y**ADR2**C**B_2) that couples to G protein G_s.

Determination of receptor internalization showed a fast uptake of [^{3}H]BK in all chimeras with C terminus exchanges distal of helix 8 (Fig. 7*C*). Solely, when the C terminus was derived from the non-internalizing B_1 bradykinin receptor (construct B2**C**B1), a quite slow and low uptake was observed. When helix 8 was substituted in addition, only the angiotensin II type 1 receptor chimera was able to function like the B_2Rwt, whereas the other chimeras displayed hardly any [^{3}H]BK uptake at all.

Taken together, these results indicate that G protein activation might require only a helix 8 of a "donor" receptor that couples to the same G protein, whereas with regard to receptor internalization the structural requirements are much more specific for a helix 8 to function properly in a different receptor environment. In contrast, the C terminus distal to helix 8 can apparently function to a great extent independently from the receptor core in the internalization process.

DISCUSSION

Helix 8 and Surface Expression—The existence of an intracellularly located 8th helix has been predicted for some GPCRs (for review, see Ref. 38) and then confirmed with the resolution of the first structure of a GPCR, the inactive rhodopsin (6). All the following structures also displayed this helix 8 (8) with the exception of CXCR4 (7). One reason for this high conservation seems to be an important role of helix 8 in stabilizing the receptor in a conformation that supports trafficking to the plasma membrane. In this respect it has been reported for several GPCRs that truncation of the C terminus up into the region of helix 8 results in a strong loss of receptor surface expression (11, 16, 17, 26). Our results with the receptor chimeras and in particular with the helix 8 point mutant K315P of the B_2R suggest that for the presumably stabilizing effect of helix 8, its specific amino acid composition is less important than maintaining its overall helical structure; all our chimeric constructs with complete transfers of helix 8 from one receptor to another were robustly expressed, but disturbance of helix 8 in the B_2R (point mutant K315P) resulted in much lower surface binding than observed for the B_2Rwt_H accompanied by strong intracellular localization (Fig. 4*E*).

As published recently, for the leukotriene B4 type 2 receptor an intact helix 8 is required for the receptor to pass the quality control process in the endoplasmic reticulum (17). In line with this report, Western blot analysis of our constructs showed that most of the newly synthesized helix 8-defective receptor proteins are apparently insufficiently glycosylated and trapped inside the cell. The observation that the helix 8-truncated construct V319* as well as construct K315P reached the surface when a membrane-permeable antagonist was added (Fig. 4*F*) suggests that the receptor molecule in the absence of a functional helix 8 can be stabilized by other means, *e.g.* an appropriate antagonist. A similar pharmacological chaperone effect was also reported for helix 8-defective mutants of the leukotriene B4 type 2 receptor (17) and for the muscarinic M_1 receptor (14), implying a potential therapeutic approach to rescue respective defective receptors.

Helix 8 and G Protein Activation—Structural data and experimental approaches suggested that after binding an agonist on the extracellular site, helix 8 might be one of the key elements

for transferring the information into the cell through becoming accessible for recruitment and activation of intracellular signaling proteins (9, 39, 40). There is now substantial experimental evidence for the involvement of helix 8 (11–13, 41–44) in the process of G protein activation, *e.g.* for several GPCRs it has been reported that truncation or deletion of helix 8 results in reduced activation (11–13, 44). Moreover, earlier studies have demonstrated that peptides derived from the helix 8 of rhodopsin (41) or the angiotensin II receptor (42, 43) can directly interact with the respective G proteins. It has also recently been reported that the modified C terminus of the G protein subunit G_q can be cross-linked to several helix 8 residues of the muscarinic M3 receptor (45). Pointing in the same direction of a direct involvement of helix 8 in the interaction with the G proteins, all our B_2R mutants with a disturbed helix 8 displayed a distinct increase in the EC_{50} values for phosphatidylinositol hydrolysis as compared with the B_2Rwt.

Helix 8 and B_2R-GRK Interaction—Whereas the publications so far have focused almost exclusively on a role of helix 8 in receptor expression or in G protein activation, it was just recently reported that helix 8 might also play a role in receptor phosphorylation and, therefore, in the interaction with GRKs (18). Mutation of basic residues in the helix 8 of the thyrotropin releasing hormone receptor resulted in reduced receptor internalization and phosphorylation that could be rescued partly by overexpression of GRK2 or -3 and fully by overexpression of GRK5 or -6. We have shown here that disturbance of helix 8 in the B_2R by truncation or mutation of a central residue to a proline (construct K315P) strongly diminished ligand-induced receptor internalization. For K315P, this effect could in part be overcome through overexpression of GRK3 (or GRK2, data not shown here) but not of the kinase-inactive variant GRK3-K220R (or of GRK5 or GRK6; data not shown). Therefore, we suggest that helix 8 of the B_2R participates directly or indirectly in specific high affinity binding of GRK3 (or GRK2). Accordingly, low affinity binding due to a defective helix 8 can be compensated for by GRK3 overexpression, which presumably results in normal phosphorylation of serine/threonine residues in the B_2R C terminus if present, thus rescuing receptor internalization. In agreement with this proposal, rescue by GRK3 was not possible for construct K315P-G327*, having a defective helix 8 and lacking the C terminus (Fig. 6*E*). On the other hand, the reduced internalization observed for the B_2R truncation G327*, caused by the absence of the receptor C terminus with its phosphorylation sites, could also be partly rescued by overexpression of GRK3 but only when GRK3 kinase activity was left functional and when the GRK3 clathrin binding site was preserved. Together with the data that mutant G327* ligand-dependently binds GRK3 (Fig. 6*A*), our results support the idea that receptor-bound GRK3 may also phosphorylation-independently mediate directly internalization via clathrin-coated pits.

Helix 8 and Arrestin Interaction—Our results show that the presence of an intact helix 8 alone without the respective C terminus is sufficient for the B_2R to associate with β-arrestin2, but not with β-arrestin1, and to generate robust internalization, although to a distinctly lesser extent than observed for the B_2Rwt. The combination of a disturbed helix 8 with C-terminal truncation (mutant K315P-G327*) completely abrogates receptor endocytosis and prevents even any rescue attempts by overexpression of β-arrestins. These data indicate that helix 8 is crucial to preferably recruit β-arrestin2 to the activated receptor and that preceding phosphorylation of the B_2R C terminus might ensure high affinity β-arrestin2 binding but is not absolutely required for a productive interaction. In contrast, β-arrestin1 is likewise strictly dependent on the B_2R C terminus, as it does not associate notably with construct G327*, at least not under the conditions applied (Fig. 5*E*). However, it may substitute for β-arrestin2 in the sequestration of the B_2R and even of G327* when β-arrestin2 levels are markedly reduced (siRNA knockdown) or with β-arrestin1 being strongly overexpressed (Fig. 5*C*). Our observed subtype-specific differences are in good agreement with a report by Zhang *et al.* (46, 47), who have previously shown that the phosphorylation-independent internalization of the δ-opioid receptor is regulated via β-arrestin2, but not via β-arrestin 1, which exclusively promotes the phosphorylation-dependent internalization.

Conclusions—Our data support the following models for the activation of the B_2R. Subsequent to an agonist-induced change in the overall receptor conformation, helix 8 becomes accessible as an interaction site not only for G proteins but also for GRK2/3 and for β-arrestin2, yet less for β-arrestin1. With regard to receptor internalization of the B_2R, helix 8 is apparently more important than the remaining C terminus with its phosphorylation sites, as ligand-induced receptor internalization was still functional in the absence of the C terminus but completely abrogated when helix 8 was disturbed by a proline point mutation. Alternatively, helix 8 does not function as a direct interaction site for G proteins, GRKs, or arrestins but indirectly as a key player in stabilizing receptor conformations that result in these interactions after binding of an agonist.

Finally, recent reports have shown that helix 8 can serve as an allosteric binding site for small molecule compounds (48, 49). Given the fact that the various functions of GPCRs are differentially affected by manipulations of helix 8, as demonstrated by our group and other researchers, targeting helix 8 as an allosteric regulatory binding site appears to be an interesting approach in the quest for more specific GPCR-related drugs.

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Alanine screening of the intracellular loops of the human bradykinin B_2 receptor – effects on receptor maintenance, G protein activation and internalization

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The bradykinin B_2 receptor is coupled to G protein $G_{q/11}$ and becomes sequestered into intracellular compartments after activation. To more closely define the receptor sequences involved in these processes and their functions, we systematically mutated all three intracellular loops (ICLs), either as point mutations or in groups of three to five amino acids to Ala, obtaining a total of 14 mutants. All constructs were stably expressed in HEK 293 cells and, with the exception of triple mutant DRY → AAA, retained the ability to specifically bind [^{3}H]bradykinin. The binding affinities at 4 or 37 °C of several mutants differed considerably from those determined for the wild-type receptor, indicating an allosteric connection between the conformation of the binding site and that of the ICLs. Mutations in ICL-1 strongly reduced surface expression without affecting G protein signaling or [^{3}H]bradykinin internalization. Two cluster mutants in the middle of ICL-2 containing basic residues displayed considerably reduced potencies, whereas two mutations in ICL-3 resulted in receptor conformations that were considered to be semi-active, based on the observation that they responded with phosphoinositide hydrolysis to compounds normally considered to be antagonists. This, and the fact that a cluster mutant at the C-terminal end of ICL-3 was signaling incompetent, hint at the involvement of ICL-2 and ICL-3 in $G_{q/11}$ activation, albeit with different functions. None of the mutants displayed reduced ligand-induced receptor internalization, indicating that the loops are not essential for this process. No conclusion could be drawn, however, with regard to the role of the DRY sequence, as the corresponding triplet mutation lacked binding capability.

The human bradykinin B_2 receptor (B_2R) mediates the effects of the nonapeptide bradykinin (BK) and of kallidin (lysyl-BK). B_2R has been reported to play a role in a number of physiological and pathophysiological situations. Its activation causes vasodilation and hypotension, increased vascular permeability and edema, or generation of pain via C fibers [1]. B_2R, which is expressed constitutively in many tissues and cultured cells, is a prototypical member of family A (rhodopsin/β-adrenergic-like receptors) of the membrane-bound

Abbreviations
B_2Rwt, bradykinin B_2 receptor wild-type; BK, bradykinin; CMV, cytomegalovirus; EC_{50}, half-maximal effective concentration; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; HEK 293, human embryonic kidney cells; ICL-1, ICL-2, ICL-3, first, second and third intracellular loops; IP, inositol phosphate; PAO, phenylarsine oxide.

G protein-coupled receptors (GPCRs), and has been shown to be coupled preferably to G protein $G_{q/11}$. Following activation, the receptor is rapidly desensitized by phosphorylation of Ser/Thr residues in its C-terminus via the actions of protein kinase C and/or G protein-coupled receptor kinases (GRKs) [2]. This leads to recruitment of arrestins and sequestration of the receptor either via clathrin-coated pits or caveolae [3,4]. Which path is actually taken may depend on the cell type and the receptor expression levels. Although the processes of signaling and regulation of human B_2R are, in general, fairly well understood, the knowledge of the molecular basis of these events at the structural level of the receptor is still very limited. For example, it is not known which determinants in the intracellular loops (ICLs) of the receptor are responsible for self-maintenance, for the recruitment and activation of the G protein, or for the initiation of the desensitization process (i.e. for relaying the information to GRKs and arrestins that the receptor is in an agonist-bound state and therefore is a target or a potential interaction partner). For other family A GPCRs, all three ICLs have been shown to participate, in one way or another, in either G protein activation or receptor sequestration [5,6]. Basic and hydrophobic residues in the second and third ICLs (ICL-2 and ICL-3, respectively) of the muscarinic receptor were identified as functionally important for G protein coupling [7,8]. ICL-1 and ICL-3 play a role in the interaction of the δ-opioid receptor with $G\alpha_{16}$ [9]. A highly conserved Pro/Ala, found in ICL-2 of most family A GPCRs, was demonstrated, by gain- and loss-of-function studies, to be a coupling site for arrestins [10]. Given that this Pro is not conserved in B_2R, however, other residues must play a role in the internalization process of this receptor. For these reasons, we decided to systematically perform Ala screening of all three ICLs of the human B_2R in order to avoid any bias with regard to which loops or residues might be crucial. This unbiased approach was also based on the high degree of conservation of B_2R sequences in the ICLs among species, which suggests structural or functional importance.

We mutated single amino acids, or clusters of three to five amino acids, in all three ICLs to Ala and expressed the resulting 14 mutants stably and isogenically (i.e. stable integration of the receptor genes at the same unique gene locus) in HEK 293 (human embryonic kidney) cells. All clones were examined for receptor self-maintenance (surface expression levels), conformation of the binding site (equilibrium dissociation constants at 4 and 37 °C), signal transduction [half-maximal effective concentration (EC_{50}) and maximal effect of inositol phosphate (IP) accumulation] and agonist-induced receptor internalization. Our results indicate a different function for the loops in G protein activation: stretches in ICL-2 seem to be responsible for the binding of G protein $G_{q/11}$ and, in ICL-3, for keeping the receptor in an inactive state, i.e. blocking/regulating the productive interaction with $G_{q/11}$. All expressed mutants were sequestered rapidly after activation, suggesting little or no involvement of the loops in the interaction with arrestins or kinases. One possible interaction site with arrestins remains, however, as mutation of the DRY sequence to triple Ala resulted in a complete loss of surface binding activity, preventing any further investigation.

Results

Ala scanning of the ICLs of B_2R

In order to identify single residues or sequences in the ICLs of human B_2R that play a role in receptor signaling and regulation, we made systematic substitutions of amino acids for Ala, either as point mutations or in clusters of three to five residues, as indicated in Fig. 1. In the first loop (ICL-1), two group mutations (termed constructs 1/1 and 1/2) and one point mutation (E66A) were made. In the second loop (ICL-2), five group mutations (termed constructs 2/1–2/5) were produced. In the third loop (ICL-3), five group mutations (termed constructs 3/1–3/5) and one point mutation (T242A) located at the C-terminal end were generated. The amino acids mutated to Ala are listed in Table 1 and their numbering is given in Fig. 2A. In accordance with Hess *et al.* [11], sequence numbering starts at the third encoded Met residue.

ICL-1 and sequences at the N-terminus of ICL-2 and at the C-terminus of ICL-3 are crucial for receptor surface expression

All receptor constructs were stably and isogenically expressed in HEK 293 cells. Employing the Flp-In system (Invitrogen, Groningen, the Netherlands), the constructs become integrated at an identical unique locus in the genome of the host cell. If this does not occur, the cells acquire no resistance to the selection antibiotic hygromycin. Despite their isogenic expression, the maximal receptor numbers (B_{max}) of the various constructs differed markedly, and several receptor mutants were expressed at significantly lower levels than the wild-type B_2R (termed B_2Rwt_H = 11.0 ± 0.7 pmol·(mg protein)$^{-1}$], even though their expression was under the control of the same cytomegalovirus (CMV) promoter (Fig. 2A, Table 1). For this reason, we also used the

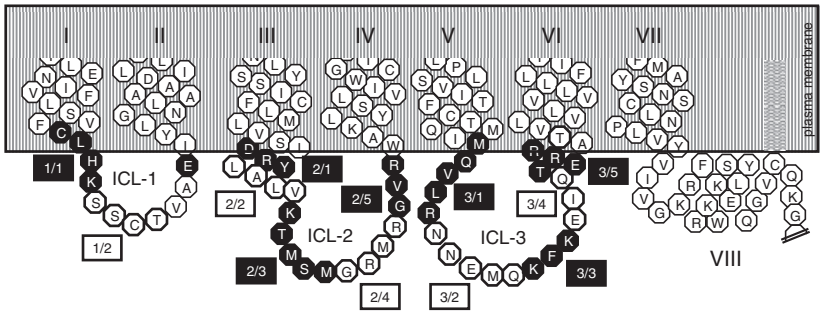

Fig. 1. Mutated sequences in human B_2R. The sequences of the ICLs, parts of the transmembrane segments (I–VII) and the proximal C-terminus containing the putative helix VIII and the palmitoylated Cys are depicted. The clusters of amino acid residues that have been mutated to Ala are depicted as black octagons or white octagons with a black edge with the respective construct name next to them. The point mutations E66A in the first loop and T242A at the end of the third loop are also indicated. The two-dimensional structure of B_2R, with the membrane border, the cytosolic extensions of the helical transmembrane domains III and VI, and the additional cytosolic helix VIII, is drawn after the structure published for inactive bovine rhodopsin [25].

Table 1. [^{3}H] binding data, and basal and BK-induced IP accumulation (NA, not applicable).

	[^{3}H]BK binding				IP accumulation		
Receptor construct	B_{max}[a] [fmol·(mg protein)$^{-1}$]	K_d(PAO/37 °C) (nM)	K_d(PAO/4 °C) (nM)	K_d ratio 37/4 °C	Basal[b]	Maximal effect[b]	EC_{50}[c] (nM)
B_2Rwt_H	11.0 ± 0.7	10.42 ± 1.56(4)	2.81 ± 0.7	3.7	2.02 ± 0.13(8)	12.55 ± 1.00	0.79 ± 0.34(4)
B_2Rwt_L	2.4 ± 0.3	8.05 ± 1.10(5)	2.02 ± 0.22	4.0	1.69 ± 0.09(4)	12.11 ± 1.22	0.67 ± 0.22(3)
1/1(CLHK)	2.3 ± 0.3	10.18 ± 0.21(3)	2.25 ± 0.37	4.5	1.76 ± 0.17(6)	14.18 ± 1.44	1.04 ± 0.21(5)
1/2(SSCT)	0.9 ± 0.1	3.21 ± 0.31(3)	0.79 ± 0.11	4.1	1.61 ± 0.05(4)	5.58 ± 0.09	0.43 ± 0.08(6)
E66A	0.7 ± 0.4	3.77 ± 0.67(3)	0.94 ± 0.29	4.0	1.81 ± 0.11(6)	6.77 ± 1.25	0.37 ± 0.07(3)
2/1(DRY)	< 0.02	NA	NA	NA	NA	NA	NA
2/2(LALV)	5.3 ± 0.2	2.96 ± 0.74(3)	1.80 ± 0.36	1.6	1.88 ± 0.13(5)	10.19 ± 1.50	2.96 ± 0.28(4)
2/3(KTMSM)	9.5 ± 0.7	9.22 ± 1.50(3)	2.80 ± 0.49	3.3	1.49 ± 0.07(3)	8.17 ± 0.72	11.66 ± 1.94(6)
2/4(GRMR)	10.2 ± 0.3	14.19 ± 1.36(3)	3.64 ± 0.17	3.9	1.40 ± 0.11(6)	9.90 ± 2.20	11.56 ± 3.02(5)
2/5(GVR)	10.3 ± 0.6	12.99 ± 0.78(4)	4.43 ± 0.57	2.9	1.59 ± 0.14(8)	14.46 ± 1.10	1.33 ± 0.22(5)
3/1(MQVLR)	5.0 ± 0.6	3.45 ± 0.53(5)	1.87 ± 0.45	1.8	1.72 ± 0.18(5)	6.91 ± 1.22	3.17 ± 0.45(3)
3/2(NNEMQ)	11.0 ± 1.3	9.54 ± 0.90(3)	5.37 ± 1.38	1.8	1.55 ± 0.12(5)	7.37 ± 0.86	3.12 ± 0.42(5)
3/3(KFK)	4.3 ± 0.7	7.93 ± 1.27(3)	2.18 ± 0.37	3.6	2.05 ± 0.17(4)	10.09 ± 1.14	3.78 ± 0.64(4)
3/4(EIQ)	13.7 ± 0.8	8.52 ± 1.74(4)	5.24 ± 1.07	1.6	1.87 ± 0.33(3)	10.53 ± 3.25	1.49 ± 0.18(4)
3/5(TERR)	0.7 ± 0.0	1.63 ± 0.50(3)	0.86 ± 0.29	1.9	1.54 ± 0.16(5)	2.07 ± 0.38	NA
T242A	12.7 ± 0.6	6.70 ± 0.93(3)	3.38 ± 0.60	2.0	1.93 ± 0.07(3)	11.37 ± 0.72	0.97 ± 0.27(4)

[a] Estimated from at least three different clones in 24 wells after incubation with 200 µL of 30 nM [^{3}H]BK on ice. [b] Total IP accumulation after 30 min of incubation in buffer with inhibitors and 50 mM LiCl at 37 °C with (maximal effect) and without (basal) 1 µM BK, expressed as the fold increase of initial total IP production (t = 0 min). The results represent the mean ± SEM of the number of experiments (given in parentheses) performed in triplicate. [c] Calculated from incubations in duplicate with 10^{-12}–10^{-5} M BK for 30 min at 37 °C in the presence of 50 mM LiCl. Results are the mean ± SEM of independent experiments (number indicated in parentheses).

Flp-In system with a weaker promoter (P_{min}), consisting of only the last 51 nucleotides of the CMV promoter, to obtain a distinctly lower expression level [2.4 ± 0.3 pmol·(mg protein)$^{-1}$] for B_2Rwt (termed B_2Rwt_L). For this construct, expression was similar to that achieved for the lower expressed mutants. B_2Rwt_L also served to estimate the degree to which the receptor expression level might influence the parameters under investigation. We have observed that compounds such as icatibant and B9430, which are generally considered to be antagonists, become partial agonists with high B_2Rwt expression levels (A. Faussner *et al.*, unpublished results), indicating that high over-expression might generate some kind of artifact. To avoid this, we additionally generated lower expressing cell lines under the control of the P_{min} promoter for some of the constructs in ICL-3 (Fig. 2A, filled bars) that otherwise, with the CMV promoter, displayed very high expression levels (Fig. 2A, Table 1) and 'antagonist-inducible' signaling (not shown).

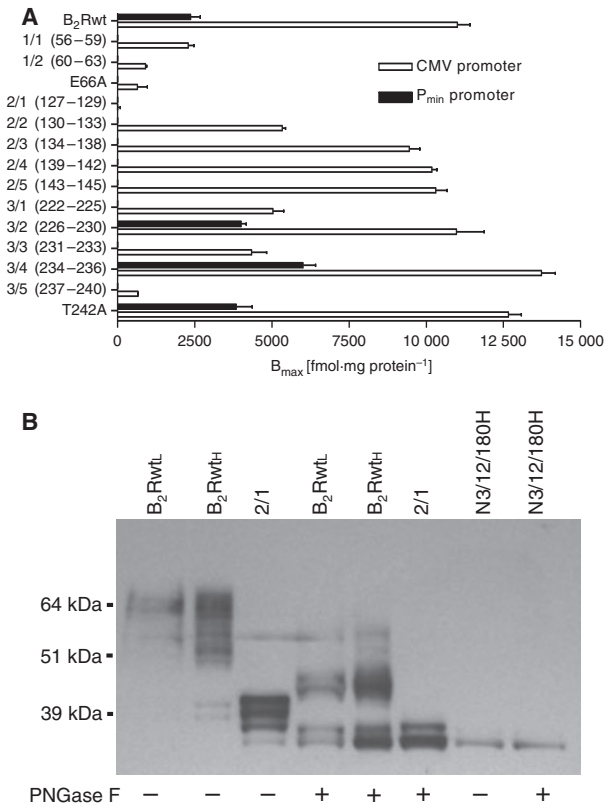

Fig. 2. Construct expression levels. (A) Maximal surface binding of [^{3}H]BK to confluent monolayers of HEK 293 cells stably and isogenically expressing the indicated constructs was estimated with approximately 30 nM [^{3}H]BK on ice, as described in Materials and methods. The data shown are the mean ± SEM of at least three clones determined in duplicate. The positions of the amino acids mutated to Ala are given in parentheses. Open columns, expression of the constructs under the control of the CMV promoter; filled columns, HA-tagged constructs under the control of the weaker P_{min} promoter. (B) Immunoblot of B_2Rwt, mutant 2/1 and the *N*-glycosylation-deficient mutant N3/12/180H. HEK 293 cells stably expressing high (B_2Rwt_H) or low (B_2Rwt_L) amounts of HA-tagged wild-type B_2R, construct 2/1 or mutant N3/12/180H were lysed in RIPA buffer, as described in Materials and methods, and treated or not with PNGase as indicated; 15 μg (only 5 μg of B_2Rwt_H) of protein was separated by SDS–PAGE and detected by western blot using a monoclonal HA antibody. The relative molecular masses of standard proteins are indicated on the left side in kilodaltons. The blot shown is representative of two experiments.

Of the constructs with Ala substitution in the sequence of ICL-1, cluster mutant 1/2 and point mutant E66A displayed particularly low binding activity, with less than 7% of that obtained for B_2Rwt_H (Fig. 2A). Exchange of the highly conserved DRY sequence located at the transition of the cytosolic extension of helix III and the N-terminus of ICL-2 for three Ala residues resulted in a construct that did not bind ligand. Figure 2B shows the immunoblot of hemagglutinin (HA)-tagged mutant 2/1 in comparison with those of B_2Rwt_H and B_2Rwt_L. For both wild-type cell lines, several bands were detected between 50 and 65 kDa with densities that largely reflected their relative expression levels, and two weaker bands at 42 and 39 kDa. Mutant 2/1, in contrast, displayed only strong bands at 42 and 39 kDa and two weaker ones at 36 and 33 kDa. An unusual migration behavior has been reported for B_2R in SDS–PAGE [12,13]. Nevertheless, the possibility remained that a lack of glycosylation caused the major bands of mutant 2/1 to run at, or below, the masses calculated for the B_2R amino acid sequence (approximately 40 kDa). However, as observed for both the high- and low-expressed B_2R wild-types, the bands of mutant 2/1 still displayed a clear shift to lower masses after enzymatic deglycosylation treatment (Fig. 2B). After deglycosylation, the major bands of construct 2/1 corresponded to the mass of the *N*-glycosylation-deficient mutant N3/12/180H. These results suggest that this receptor mutant is expressed and glycosylated, but nevertheless is unable to reach the plasma membrane. In fact, a fusion protein of construct 2/1 with enhanced green fluorescent protein joined to the C-terminus demonstrated strong intracellular expression and also did not display any specific surface [^{3}H]BK binding activity (not shown). All other constructs with mutations made in ICL-2 were strongly expressed. The mutants made in ICL-3 all revealed high expression levels, with the exception of mutant 3/5, positioned at the C-terminus of ICL-3, which displayed only 6% of that obtained for B_2Rwt_H.

These results demonstrate that the ICLs, in particular ICL-1, the conserved DRY sequence at the N-terminus of ICL-2 and the C-terminus of ICL-3, play a crucial role in the self-maintenance of the receptor, and that small changes in amino acid composition of the sequences can significantly affect the number of receptors reaching the cell surface. So far, however, our data allow no conclusion to be drawn (except for construct 2/1) on the cause of the different expression levels observed.

Mutations in ICLs affect the receptor ligand binding site

The receptor equilibrium binding affinity (K_d) reflects the conformation of the extracellular ligand binding site. Differences in the affinities displayed by the expressed mutant constructs may therefore indicate different preferences in coupling to intracellular proteins, such as G proteins, arrestins or receptor kinases,

because such interactions can also affect the overall receptor conformation, including the binding site. Most GPCRs respond to an agonist at higher temperatures with receptor sequestration. As a consequence, K_d values are usually determined either in intact cells on ice or at a suitable temperature (4–37 °C) in whole-cell lysates or membrane preparations. One disadvantage of the former approach is that receptors barely signal at 4 °C. By contrast, a disadvantage of the latter approach is that interacting proteins that become recruited from the cytosol after receptor activation may either be too diluted (whole-cell lysates) or be no longer present at all (membrane preparations). Therefore, as an alternative, we have established a method [14] whereby, through the inhibition of receptor sequestration by pretreatment of the cells with phenylarsine oxide (PAO), we can determine the K_d value at 37 °C (and at 4 °C) in whole intact cells with all cytosolic proteins present.

At 37 °C, B_2Rwt_L displayed an affinity for [^{3}H]BK of 8.05 ± 1.10 nM. This was increased to 2.02 ± 0.22 nM (n = 5) when incubations were performed on ice, corresponding to an approximately four-fold increase in affinity (Fig. 3, Table 1). A similar pattern was seen for B_2Rwt_H, although both affinities were slightly lower (10.42 ± 1.56 and 2.81 ± 0.7 nM, respectively). Mutants 1/2 and E66A showed a higher affinity than B_2Rwt_L at 37 °C (3.21 ± 0.31 and 3.77 ± 0.67 nM, respectively), but also showed a four-fold shift to higher affinity when incubated on ice (0.79 ± 0.11 and 0.94 ± 0.29 nM, respectively), thus retaining their higher affinity relative to B_2Rwt at both temperatures. In contrast, mutant 2/2 in ICL-2 displayed a high affinity at 37 °C, but exhibited almost no shift to higher affinity on ice (2.96 ± 0.74 nM at 37 °C versus 1.8 ± 0.36 nM at 4 °C), suggesting that this mutant is in a high-affinity state at 37 °C. The other mutants in ICL-2 displayed affinity increases at 4 °C relative to 37 °C that were similar to those observed for B_2Rwt (Fig. 3).

With the exception of mutant 3/3, all constructs generated in ICL-3 displayed a binding behavior clearly different from that of B_2Rwt. Although the mutations located in the middle of ICL-3 (constructs 3/2 and 3/4) and T242A showed affinities at 37 °C that were similar to those determined for B_2Rwt, they did not respond to incubation at 4 °C with an increase in affinity as pronounced as that seen for B_2Rwt, displaying a shift of less than two-fold. The constructs at either the N-terminal (mutant 3/1) or C-terminal (mutant 3/5) end of ICL-3 exhibited a high affinity at 37 °C and at 4 °C (Fig. 3, Table 1).

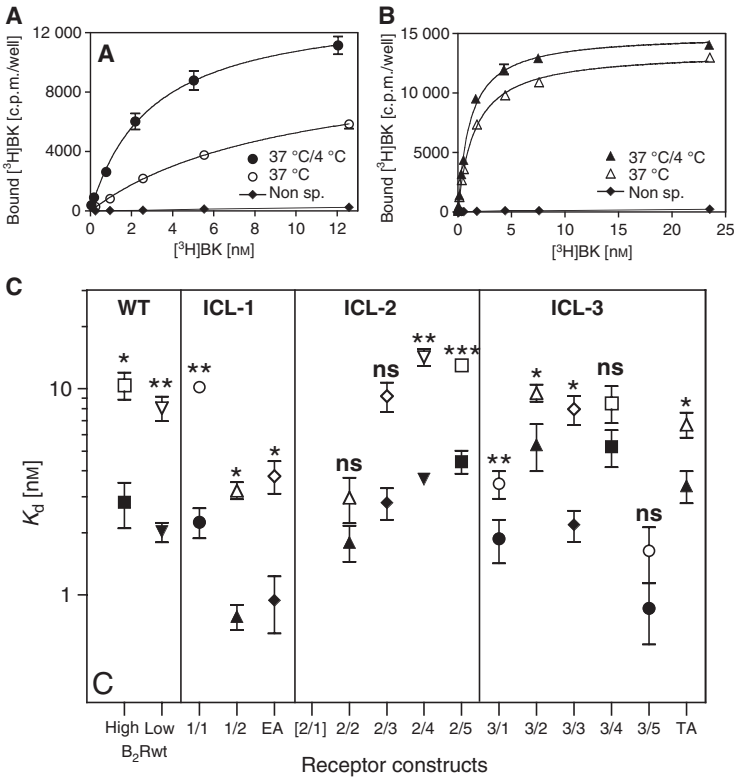

Fig. 3. Equilibrium dissociation constants K_d at 37 and 4 °C. Binding of [^{3}H]BK (0.01–30 nM) to HEK 293 cells stably expressing the indicated constructs was determined at 37 and 4 °C after inhibition of receptor sequestration by pretreatment of the cells with 100 μM PAO, as described in Materials and methods. Two representative binding curves are shown: (A) construct 1/1; (B) construct 2/2. (C) K_d values of all constructs as mean ± SEM of at least three experiments (results also given in Table 1). Open symbols, K_d values at 37 °C; filled symbols, K_d values at 4 °C. Note the logarithmic scale of the y-axis. Comparison between K_d values at 37 and 4 °C: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant.

As these differences in binding affinity could not be caused by a direct effect of the mutations on [^{3}H]BK binding, they must be induced allosterically through changes in the overall structure of the receptor. Thus, these data demonstrate the connection between the structure of ICLs and that of the binding site, implying reciprocally that changes at the binding site through binding of an (inverse) agonist could also induce conformational changes in the ICLs, as required for signal transduction.

Basal activity and stimulated accumulation of IPs

Stimulation of B_2Rwt leads to activation of phospholipase C via G protein $G_{q/11}$, resulting in the release of inositol trisphosphate. In order to determine the effects of the loop mutations on the interaction of the receptor with $G_{q/11}$, we measured the accumulation of IPs in the presence of 50 mM LiCl with and without stimulation by BK for 30 min. The fact that some of the mutants did not show a strong difference in their affinities at 37 and 4 °C (see Fig. 3) suggests that they might be in a permanently higher affinity state, i.e. have a semi-active conformation. If so, they could either exhibit a higher basal activity or display a strong signal in response to even poor partial agonists. As mentioned previously, the pseudopeptides icatibant (also known as Hoe140 or Je049) and B9430 were partial agonists at B_2Rwt_H, but were not able to elicit an IP response when the receptors were expressed at a lower level, comparable with B_2Rwt_L (Fig. 4). Thus, these drugs provide a tool for the identification of semi-active mutants, provided that these constructs are expressed at lower levels. To meet this requirement, we expressed the constructs also under the control of the weaker P_{min} promoter, in case we observed a response to B9430 and icatibant at expression levels higher than 7 pmol receptor·(mg protein)$^{-1}$. As this was the case for the constructs 3/2, 3/4 and T242A (data not shown), we used them for IP experiments at the lower expression levels, as depicted in Fig. 2A.

The activation of most constructs by 1 μM BK induced an 8–15-fold increase over basal IP, determined as the amount of IP in cells kept on ice (Fig. 4,

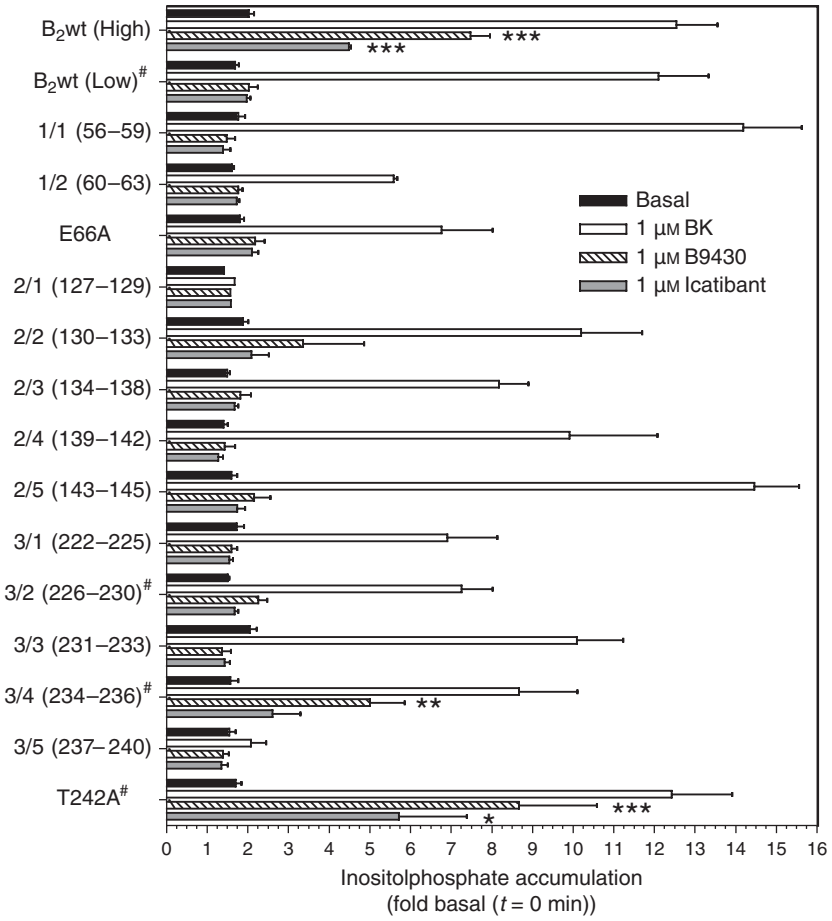

Fig. 4. Basal and stimulated accumulation of total IPs. Cells in 12-well plates were preincubated overnight with 0.5 μCi [^{3}H]inositol. IP accumulation (basal and stimulated) in the presence of 50 mM LiCl after incubation for 30 min at 37 °C with 1 μM of the indicated peptides was determined as described in Materials and methods. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. The results are presented as the fold increase over the IP content of identically treated control cells that had remained on ice. Basal (black columns), BK (open columns), B9430 (hatched columns), icatibant (grey columns). #Use of cells expressing smaller amounts of the constructs under the control of the P_{min} promoter [mutant 3/2, 4.0 ± 0.2 pmol·(mg protein) $^{-1}$; mutant 3/4, 6.0 ± 0.4 pmol·(mg protein) $^{-1}$; mutant T242A, 3.8 ± 0.5 pmol·(mg protein) $^{-1}$]. Comparison between basal and stimulated IP accumulation: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

open columns; Table 1). In our experimental set-up, there appears to be no direct linear correlation between the induced accumulation of IPs and the amount of expressed receptors, as demonstrated by the example of B_2Rwt (see Table 1), where an almost five-fold higher expression of surface receptors [11.0 versus 2.4 pmol·(mg protein)$^{-1}$] did not result in a significantly higher IP response (12.55 ± 1.00-fold versus 12.11 ± 1.22-fold over the basal level). This suggests that, at these levels, the receptor number is not limiting for the maximal response, and that most of the over-expressed receptors are not directly coupled to signal transduction. To avoid an over-interpretation of the data, we attempted only a semi-quantitative evaluation of the IP data. Some results, however, require some comment. For example, all mutants made in ICL-1 (1/1, 1/2, E66A) had an apparently strong signal relative to their expression level, particularly mutant 1/2 and point mutant E66A. A similar signal, however, was obtained when an inducible B_2Rwt was expressed at the same low levels using the Flp-In/Trex expression system (A. Faussner *et al.*, unpublished results). In contrast, mutant 3/5, which expressed at the same low levels, showed almost no response at all, suggesting a pivotal role for this sequence (or part of it) in the coupling to and/or activation of $G_{q/11}$.

When stimulated with 1 μM of the partial agonists B9430 and icatibant, most of the mutants did not respond with increased IP accumulation. However, in two mutants in ICL-3 (3/4 and T242A), exposure to these compounds resulted in a significant increase in accumulated IPs (Fig. 4), suggesting that these mutations result in a semi-active receptor conformation with regard to $G_{q/11}$ activation. The mutated sequences therefore apparently contribute to keeping the receptor in an inactive state, but are not solely responsible for regulating the activation state, as none of these mutations resulted in increased basal, agonist-independent activity of the receptor (Fig. 4, Table 1).

EC_{50} of IP accumulation

There was no significant difference in the EC_{50} values obtained with B_2Rwt expressed at two different levels, demonstrating that, at these levels, the efficiency of BK is independent of the number of receptors (Fig. 5, Table 1). In all the mutants made in ICL-1, BK displayed efficiencies similar (1/1) or apparently even higher (1/2, E66A) than those observed in B_2Rwt, in agreement with their higher binding affinities at 37 °C (see Fig. 3).

Of the constructs generated in ICL-2, mutants 2/3 and 2/4 showed a strongly increased EC_{50} value (approximately 15-fold) when compared with B_2Rwt (Fig. 5, Table 1). As these constructs displayed maximal responses similar to that of B_2Rwt (see Fig. 4), these results indicate that constructs 2/3 and 2/4 display weaker coupling of $G_{q/11}$, but do not lack in general the ability to fully activate the G protein.

All cluster mutations of ICL-3, but not point mutation T242A, exhibited a tendency to reduced efficiency, but this failed to achieve statistical significance. These results, combined with the lower maximal responses (Fig. 4), suggest that the sequences at the N-terminus and in the middle of ICL-3 may participate in the

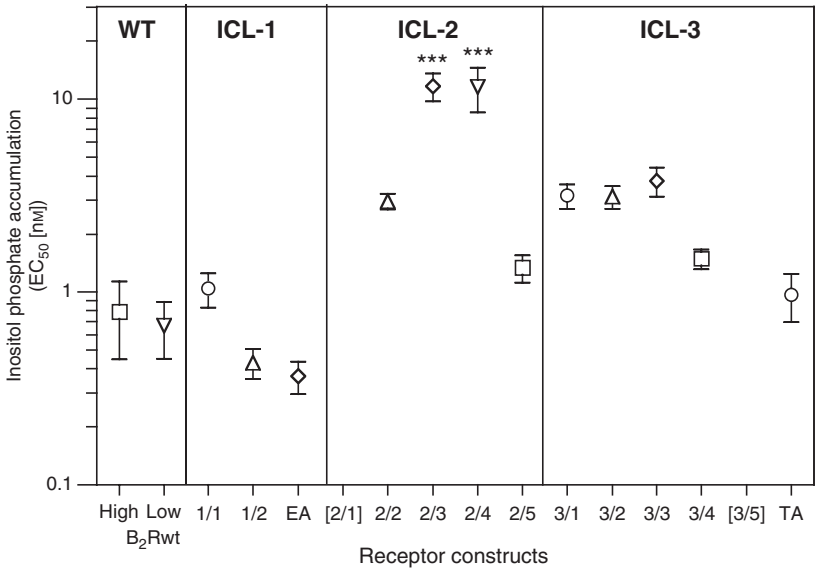

Fig. 5. EC50 values of IP accumulation. Cells were treated and incubated as described in the legend of Fig. 4 with increasing concentrations of BK (10^{-12}–10^{-5} M) for 30 min at 37 °C, and the determination of total IPs was performed as described in Materials and methods. EC_{50} is given as the mean ± SEM of the number of experiments indicated in Table 1. Note the logarithmic scale of the *y*-axis. Comparison with EC_{50} values of both high and low B_2Rwt: ***$P < 0.001$.

coupling and activation of $G_{q/11}$. As a result of its lack of activity, no EC_{50} value could be obtained for mutant 3/5.

Internalization of [^{3}H]BK

After stimulation, most GPCRs, including B_2Rwt, become sequestered to compartments within the cell. Recent publications have indicated that the ICLs of GPCRs might not only be involved in the interaction with their cognate G proteins, but may also serve, together with (phosphorylated) Ser/Thr residues in the C-terminal tail, as contact sites for arrestins and GRKs**.** Thus, these loops may also contribute to receptor internalization [10,15]. In addition, diminished or increased ability to interact with the cognate G protein(s), or a changed capability to activate them, might also affect this process through steric hindrance. Therefore, we also examined the capabilities of the various constructs to internalize [^{3}H]BK. Having demonstrated recently that the internalization decreases when too many receptors are occupied in cells with high receptor expression [16], we took care to use nonsaturating concentrations of less than 2 nM [^{3}H]BK. Under these conditions, none of the constructs exhibited significantly slower internalization than that observed for B_2Rwt (Fig. 6). This suggests that the amino acid residues involved are not of pivotal significance for internalization. As a result of its lack of surface binding activity, no results could be obtained for mutant 2/1, i.e. participation of the highly conserved DRY sequence in the internalization process cannot be excluded by our data.

Discussion

The goal of the present study was to identify residues and regions in the intracellular domains of human B_2R that play a major role in its signal transduction and sequestration processes – specifically, regions that are involved in interactions with G proteins, receptor kinases or arrestins. To this end**,** and in order to avoid any bias by focusing only on highly conserved residues, we set out to systematically mutate all three ICLs. To reduce the amount of constructs, we started with the generation of 12 cluster mutations (three to five amino acids) and two point mutants. Our use of the Flp-In system (Invitrogen) guaranteed stable isogenic expression, and thus allowed direct comparison of the expression levels of the various constructs without having to take into account a possible different insertion into the genome of the host cell line affecting the expression level *per se*. In total, only one mutant (construct 2/1) of the 14 constructs displayed no binding activity at all; several others exhibited low expression, but still signaled well, and only one mutant (construct 3/5) did not signal at all, despite detectable binding.

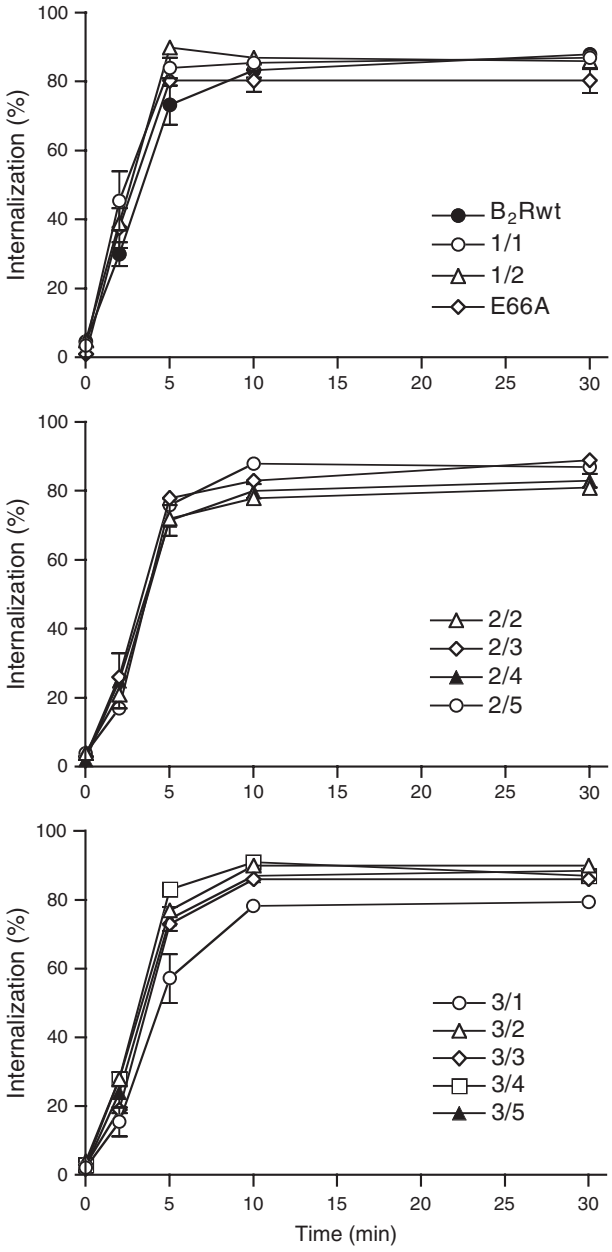

Fig. 6. Internalization of [^{3}H]BK. Cells expressing B_2Rwt or the indicated receptor constructs were preincubated with 2 nM [^{3}H]BK for 90 min on ice. Internalization was started by warming the plates to 37 °C in a water bath. At the given time points, surface-bound and internalized [^{3}H]BK were determined by acetic acid treatment, as described in Materials and methods. Internalization is given as a percentage of total bound [^{3}H]BK (surface plus internalized [^{3}H]BK). Data points represent the mean ± SEM of at least three experiments performed in duplicate or triplicate.

All mutations in ICL-1 resulted in a strong decrease in binding without affecting either receptor signaling or sequestration. These results are in agreement with reports on rat B_2R [17] as well as rhodopsin [18], indicating that ICL-1 forms a tight bend, the disturbance of which strongly affects the receptor expression level. Thus, ICL-1 is important for the maintenance of the overall receptor structure and stability, but is apparently of no functional importance otherwise. Regions in ICL-2 and ICL-3 appear to play a substantial role in the correct processing and trafficking of the receptor, as demonstrated by the overall lower surface expression levels of the mutants generated in these loops. Constructs 3/5 and 2/1, in particular, displayed little or no surface binding, respectively. Each of these two regions includes negatively and positively charged amino acids that might be crucial for correct folding. Of particular interest are the two residues R128 in sequence 2/1 and E238 in sequence 3/5 (Figs 1 and 7) that are highly conserved in many family A GPCRs. It has been postulated that they form an 'ionic lock' that upholds the inactive state of the receptor by stabilizing it [19]. A similar stabilizing role in B_2R might explain why mutation of the regions containing these residues strongly affects surface expression.

Our results indicate that ICL-2 and ICL-3 are strongly involved in the interaction with $G_{q/11}$, but in different ways. Mutations in ICL-2 resulted in a clear reduction in signaling potency (more than 10-fold), but not in a significantly reduced maximal response, suggesting that the sequences mutated participate in the coupling to $G_{q/11}$ but not in its activation. The notion of impaired coupling of ICL-2 mutants is also supported by the fact that, despite their high expression levels, mutants 2/3–2/5 could not be activated by B9430 or icatibant, in contrast with B_2Rwt_H. In addition, they also displayed the lowest basal activities of all constructs, hinting at an inverse agonistic effect of these mutations regarding the activation of $G_{q/11}$. The cluster mutation 3/4 and point mutation T242A in ICL-3, in contrast, resulted in semi-active receptor conformations, as these expressed constructs gave a clear response to these otherwise poor partial agonists. Our observation that semi-active conformations do not necessarily result in increased basal activity (Fig. 4) has also been reported for bovine rhodopsin, where the mutation of Tyr to Ala in the highly conserved NPXXY sequence did not result in increased basal activity, but turned a poor agonist into a potent one [20].

Looking at the affinities of the mutants at 37 and 4 °C, only three constructs (1/1, 2/3 and 3/3) showed binding characteristics comparable with those observed for B_2Rwt. All other mutants differed significantly at either 4 or 37 °C, demonstrating that mutations in ICLs also affect the conformation of the extracellular binding site. Whether these different K_d values are inherent properties of the mutants caused by a change in the overall receptor conformation, or reflect modified interactions with cytosolic proteins stabilizing certain receptor conformers, will require further study. A strong reduction in the affinity shift [K_d(37 °C)/K_d(4 °C) ≤ 2] apparently points to a semi-active receptor conformation, as two constructs with a weak affinity shift (3/4 and T242A; Fig. 3, Table 1) responded well to poor partial agonists with the accumulation of IPs (Fig. 4). This indicates a role for the respective sequences in maintaining the receptor in an inactive state and preventing unwanted interaction and activation of $G_{q/11}$. Intriguingly, homology modeling of B_2Rwt using the Expasy Proteomics Server software DEEP VIEW, employing the structure of inactive rhodopsin (Protein Data Bank access code PDB 1U19) as a template, resulted in a three-dimensional structure that displayed the regions relevant for semi-activity clearly separated from those related to potency reduction (Fig. 7). These latter sequence types, such as the mutations in constructs 2/4 and 2/5, apparently contribute to the coupling of the receptor to G protein $G_{q/11}$. A look at the acidic, negatively charged surface of G protein heterotrimers [21] might explain these results. These regions contain positively charged residues (K134 in 2/3 and R140/R142 in 2/4), whereas one mutation resulting in a semi-active conformation is missing a negative charge (E234 in 3/4) that might serve to repel, to a certain degree, the negatively charged G proteins in the inactive state.

Although a role of ICLs in receptor internalization has been reported for other family A GPCRs [10,22], no significant differences were observed between B_2Rwt and the loop mutants regarding their internalization of [^{3}H]BK. These results are consistent with our previous observation that the intracellular C-terminus is crucial for ligand-induced receptor internalization [23]. Swapping the C-terminal tails between B_1R and B_2R was sufficient to transfer the capability to undergo rapid ligand-induced receptor internalization to B_1R, a receptor which, in its wild-type state, does not become internalized in response to agonist stimulation [24]. However, we cannot exclude the possibility that the effects of the mutations on interactions with either receptor kinases or arrestins were not sufficiently strong to be detected under the conditions used. Alternatively, it is possible that the region pivotal for these interactions is the DRY sequence that could not be investigated in this regard, because of a lack of surface

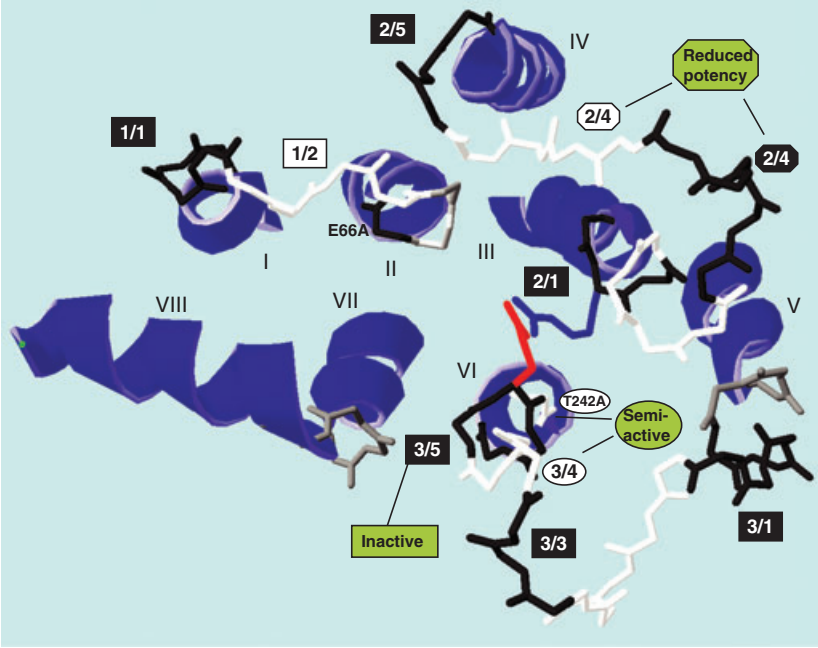

Fig. 7. Position of mutations and annotation of their effects in a computational model of B_2R as seen from the cytosol. The structure was generated with SWISS-model [26] based on the crystal structure of bovine rhodopsin in its inactive form (PDB 1U19A [27]). Dark blue, cytosolic ends of the seven transmembrane helices I–VII and cytosolic helix VIII; point and cluster mutants are depicted in black and white, as also shown in Fig. 1 (only α-carbon chain, no side-chains shown, with the exception of R128 in blue and E238 in red); grey, amino acids not mutated outside of helices; green boxes, effect of indicated mutations on receptor properties.

binding of the triple mutant. Results with the CXCR5 receptor do, indeed, suggest binding of arrestins to the region of the DRY sequence [15]. The fact that construct 3/5 has minimal capacity to stimulate $G_{q/11}$ (Fig. 4), but nevertheless becomes sequestered as quickly as B_2Rwt (Fig. 6), indicates that G protein activation and receptor internalization are two independent processes, i.e. sequestration of the receptor is not a consequence of a prior activation of the G protein.

In summary, our results show that changes in ICL-1 strongly affect receptor surface expression, but not receptor signaling or receptor sequestration. Even more important for receptor maintenance are the DRY sequence at the N-terminus of ICL-2 and the TERR sequence at the C-terminus of ICL-3, as cluster mutations here complete abolish (construct 2/1) or strongly reduce (construct 3/5) surface receptor expression. Both ICL-2 and ICL-3 are involved in the interaction with G protein $G_{q/11}$, but in different ways. Sequences in ICL-2 apparently contribute more to the coupling, and regions in ICL-3 preferentially to the activation, of $G_{q/11}$. None of the three ICLs appears to have a crucial function in the sequestration process, i.e. in the interaction with receptor kinases and/or arrestins, with the caveat that, as a result of experimental reasons, no conclusion can yet be drawn on the role of the highly conserved DRY sequence at the N-terminus of ICL-2. Our results obtained with the cluster mutations indicate that certain sequences need to be investigated in more detail, and will therefore be targeted in future studies for the generation of point mutants.

Materials and methods

Materials

Flp-In™ TREx-293 (HEK 293) cells were obtained from Invitrogen. [2,3-Prolyl-3,4-^{3}H]BK (2.96 TBq·mmol^{-1}) and myo-[2-^{3}H]inositol (0.81 TBq·mmol^{-1}) were obtained from PerkinElmer Life Sciences (Boston, MA, USA). BK was purchased from Bachem (Heidelberg, Germany). B9430 and icatibant were generous gifts from J. Stewart (Denver, CO, USA) and Jerini (Berlin, Germany), respectively. Roche (Mannheim, Germany) delivered Fugene. Poly-D-lysine, captopril, 1,10-phenanthroline and bacitracin were purchased from Aldrich (Taufkirchen, Germany). Fetal calf serum, culture media, hygromycin B and penicillin/streptomycin were obtained from PAA Laboratories (Cölbe, Germany). Primers were synthesized by Invitrogen and delivered desalted and lyophilized.

Gene mutagenesis, expression and cell culture

Standard PCR techniques with primers designed accordingly and the B_2Rwt gene as template were used to generate point- or cluster-mutated versions of B_2Rwt. In each case, successful mutation was verified by sequencing (Medigenomix, Martinsried, Germany). The coding sequences of

B_2Rwt and all mutants started with the third encoded Met [11], and were cloned into the *Hind*III and *Xho*I sites of the pcDNA5/FRT vector (Invitrogen). Most of the receptor sequences were preceded at the N-terminus by a double tag (MGRSHHHHHHGYPYDVPDYAGS), with the last two amino acids (Gly-Ser) of the tag being generated by the insertion of a *Bam*HI site. A few constructs were tagged with a single HA tag (MGYPYDVPDYAGS) wherever indicated. The nature of the tags did not influence the pharmacological properties of the constructs. For stable expression of the constructs, we used the Flp-In system from Invitrogen, in which the vector containing the gene of interest is inserted at a unique locus into the genome of the special host cell line Flp-In™ TREx-293 (HEK 293) through the transient concomitant expression of the recombinase pOG44. HEK 293 cells, cultivated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin, were transfected using the transfection reagent Fugene (Roche) following the instructions of the manufacturer. Single stably expressing clones resulted after selection with 250 $\mu g \cdot mL^{-1}$ hygromycin B. For experiments requiring repeated rinsing of the cells, poly-D-lysine-treated (0.01% in NaCl/P_i) cell culture dishes were used to ensure adherence.

Equilibrium binding experiments at 37 and 4 °C

For the determination of the equilibrium binding affinity constant K_d at 4 °C and, in particular, at 37 °C, receptor sequestration was inhibited by pretreatment of the cell monolayers in 48 wells with 100 μM PAO in incubation buffer (40 mM Pipes, 109 mM NaCl, 5 mM KCl, 0.1% glucose, 0.05% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) for 5 min at 37 °C, as published previously [14]. Thereafter, the cells were rinsed three times with ice-cold NaCl/P_i, 0.2 mL of incubation buffer with degradation inhibitors (2 mM bacitracin, 0.8 mM 1,10-phenanthroline and 100 μM captopril) containing increasing concentrations of [^{3}H]BK (from approximately 0.01 to 30 nM) was added and the cells were immediately warmed to 37 °C in a water bath. For the determination of the affinities at 37 °C, the incubation was stopped after 30 min by placing the trays on ice and rinsing the cells four times with ice-cold NaCl/P_i. Surface-bound [^{3}H]BK (> 95% of totally bound radioactivity in cells pretreated with PAO) was dissociated by a 10 min incubation with 0.2 mL of an ice-cold dissociation solution (0.2 M acetic acid–0.5 M NaCl, pH 2.7), transferred to a scintillation vial and counted in a β-counter after the addition of scintillation fluid. For determination of the affinities at 4 °C, the initial 30 min incubation at 37 °C was followed by an additional incubation on ice. After 90 min, these cells were also rinsed with ice-cold NaCl/P_i and [^{3}H]BK binding was measured as described above. Nonspecific binding was determined in the presence of 5 μM of unlabeled BK and subtracted from the total binding determined with [^{3}H]BK alone to calculate the specific binding.

Determination of total IP release

Monolayers of stably transfected HEK 293 cells on 12 wells were incubated overnight with 0.5 mL complete medium containing 1 μCi [^{3}H]inositol$\cdot mL^{-1}$. The cells were washed twice with NaCl/P_i and pre-incubated for 90 min on ice in incubation buffer supplemented with 50 mM LiCl with or without the addition of increasing concentrations (10^{-12}–10^{-5} M) of BK. Stimulation was started by placing the cells in a water bath at 37 °C and continued for 30 min. The accumulation of total IPs was terminated by exchanging the buffer for 0.75 mL of ice-cold 20 mM formic acid solution. After 30 min on ice, another 0.75 mL of formic acid solution, followed by 0.2 mL of a 3% ammonium hydroxide solution, were added. The mixture was applied to AG 1-X8 anion exchange columns (Biorad, Munich, Germany; 2 mL volume). The columns were washed with 1 mL of 1.8% ammonium hydroxide and 9 mL of 60 mM sodium formate/5 mM tetraborate buffer, followed by 0.5 mL of 4 M ammonium formate/0.2 M formic acid. Total IPs were finally eluted in 2 mL of the latter buffer and counted in a β-counter after the addition of scintillation liquid.

Immunoblotting

Monolayers in six-well trays were washed three times with NaCl/P_i and solubilized in RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, pH 7.5) including 0.5 mM Pefabloc SC and 10 μM each of 1,10-phenathroline, aprotinin, leupeptin and pepstatin A for 30 min at 4 °C with gentle agitation. The lysate was centrifuged at 6240 ***g*** for 15 min at 4 °C. The supernatant (protein concentration, ~1 $mg \cdot mL^{-1}$) was treated with PNGase (Roche, Mannheim, Germany) for 2 h at 37 °C as indicated, mixed with Laemmli buffer and incubated for 10 min at 70 °C. Following electrophoresis (~15 μg total protein per lane unless stated otherwise) on 4–12% SDS–polyacrylamide gels, the fractionated proteins were electroblotted onto 0.45 μm nitrocellulose. The membrane was blocked for 1 h at 4 °C with 5% milk powder in washing buffer (Tris-buffered saline, pH 7.5, 0.1% Tween 20), and incubated overnight with primary anti-HA high-affinity IgG (1 : 2000) added in fresh blocking buffer. After washing the membrane three times, each for 10 min, the secondary peroxidase-labeled anti-rat Ig (1 : 2000) was added for 1 h at room temperature in blocking buffer. Finally, the membrane was washed again three times, each for 10 min, before antibody binding was detected using western blot Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA, USA).

[^{3}H]BK internalization

Cells on multiwell plates (24 well/48 well) were rinsed three times with NaCl/P_i and incubated with 0.2 mL of

approximately 2 nM [^{3}H]BK in incubation buffer for 90 min on ice in order to obtain equilibrium binding. [^{3}H]BK internalization was started by placing the plates in a water bath at 37 °C. The internalization process was stopped at the indicated times by putting the plates back on ice and washing the cells four times with ice-cold NaCl/P_i. Subsequently, surface-bound [^{3}H]BK was dissociated by incubating the cell monolayers for 10 min with 0.2 mL of ice-cold dissociation solution. The remaining monolayer with internalized [^{3}H]BK was lysed in 0.2 mL of 0.3 M NaOH and transferred with another 0.2 mL water to a scintillation vial. The radioactivity of both samples was determined in a β-counter after the addition of scintillation fluid. Nonreceptor-mediated [^{3}H]BK surface binding and internalization were determined in the presence of 5 μM unlabeled BK and subtracted from total binding to calculate the specific values. Internalization was expressed as the amount of internalized [^{3}H]BK as a percentage of the combined amounts of internalized and surface-bound [^{3}H]BK.

Protein determination

Total protein was quantified with the Micro BCA Protein assay reagent kit from Pierce (Rockford, IL, USA) using BSA as standard.

Data analysis

All data analysis was performed using GRAPHPAD PRISM for Macintosh, Version 4.0c (GraphPad Software, Inc., San Diego, CA, USA). Data were assessed by appropriate analysis of variance (ANOVA), with subsequent *post hoc* analysis using the Student–Newman–Keuls test. Alternatively, paired *t*-tests were used as indicated.

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