Aus dem Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten
des Klinikums der Ludwig-Maximilians-Universität München

Direktor: Univ.-Prof. Dr. med. Christian Weber

Die Bradykinin B_1 und B_2 Rezeptoren als Modell für die Untersuchung der Regulation G-Protein-gekoppelter Rezeptoren

Dissertation
zum Erwerb des Doktorgrades der Naturwissenschaften
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Jasmin Leschner
aus München

2012

Gedruckt mit Genehmigung der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

Betreuer:	Priv.-Doz. Dr. Alexander Faußner
Zweitgutachter:	Priv.-Doz. Dr. Harald Mückter
Dekan:	Prof. Dr. med. Dr. h.c. Maximilian Reiser, FACR, FRCR

Tag der mündlichen Prüfung: 05.12.2013

Für meine Familie

1 Zusammenfassung

Die Familie A der G-Protein-gekoppelten Rezeptoren (GPCRs) bildet die größte und vielfältigste aller Transmembranrezeptorfamilien. Ihre Mitglieder spielen eine wesentliche Rolle in fast allen (patho)physiologischen Prozessen. Nach Agonistenbindung aktivieren GPCRs, wie ihr Name andeutet, heterotrimere G-Proteine aber auch G-Protein-unabhängige Signalwege. Die verschiedenen aktiven G-Proteinuntereinheiten (Gα-GTP und βγ) induzieren nach Dissoziation vom Rezeptor entsprechende Signalkaskaden z.B. über Phospholipase A und Cβ. Um eine Fehlregulation zellulärer Prozesse z.B. durch „Überstimulation" zu verhindern, unterliegen GPCRs strengen Regulationsmechanismen, die ihre Fähigkeit zur Signaltransduktion und ihre Verfügbarkeit an der Zelloberfläche bestimmen.

Die Bradykininrezeptoren B_1 und B_2 (B_1R, B_2R) gehören zur Familie A der GPCRs, also zu den Rhodopsin-ähnlichen GPCRs, und werden durch die pro-inflammatorischen Peptide desArg^9-Bradykinin/desArg^{10}-Kallidin (DABK/DAK) bzw. Bradykinin (BK)/Kallidin aktiviert. Im Gegensatz zum konstitutiv exprimierten B_2R, der nach Stimulation schnell desensitisiert und internalisiert wird, erfolgt eine B_1R-Expression fast ausschließlich unter pathophysiologischen Bedingungen über Induktion durch Zytokine. Nach Stimulation wird der B_1R nicht internalisiert, sondern verbleibt an der Zelloberfläche. Beide Rezeptoren koppeln sowohl an $G\alpha_{q/11}$ als auch an $G\alpha_i$ und aktivieren somit weitgehend identische Signalwege [vor allem Phospholipase Cβ (PLCβ) und „mitogen activated protein kinase" (MAPK)-Kaskaden]. Durch ihre - besonders im Hinblick auf ihre Internalisierungs-eigenschaften - konträre Regulation, stellen die Bradykininrezeptoren ein interessantes Modell zur Untersuchung regulatorischer Mechanismen und deren Einflüsse auf die Signalübertragung von GPCRs dar.

Beide Bradykininrezeptoren spielen bei inflammatorischen Prozessen eine Rolle. Sie fördern die Ausschüttung pro-inflammatorischer Zytokine und rekrutieren Immunzellen. Während entzündlicher Ereignisse kommt es zu erhöhter Zytokinfreisetzung z.B. von Interleukin-1β (IL-1β) und dadurch zur *de novo* Synthese von B_1R. Pro-inflammatorische Zytokine wie IL-1β, die zur B_1R-Expression führen, induzieren unter anderem aber auch einen Anstieg der Körpertemperatur (Fieber), eine häufige Begleiterscheinung inflammatorischer Vorgänge. Trotz des bekannten Zusammenhangs zwischen Inflammation und erhöhter Temperatur war über den Einfluss eines Temperaturanstiegs auf Membranrezeptoren und ihre Signalvermittlung auf zellulärer Ebene bisher nur sehr wenig bekannt.

In dieser Arbeit wurde - unseres Wissens nach - erstmals auf die Temperatur als regulatorische Komponente für GPCR-vermittelte Signalübertragung eingegangen. Am Beispiel der Bradykininrezeptoren wurde gezeigt, dass die Stärke der Signalübertragung von GPCRs signifikant durch eine Temperaturerhöhung von 37°C auf 41°C beeinflusst werden kann. Hierbei war jedoch zwischen einer Temperaturabhängigkeit des Signalwegs selbst und einer rezeptorspezifischen Temperatursensitivität zu unterscheiden. So wurde die Aktivierung von ERK1/2 unter pathophysiologisch erhöhter Temperatur (41°C; normale Körpertemperatur: 37°C) signifikant gesteigert, unabhängig davon ob sie durch B_1 oder B_2 Rezeptoren stimuliert wurde. Die gesteigerte Aktivität PLCβ-vermittelter Signalkaskaden bei 41°C konnte hingegen auf eine nur für den B_1R spezifische Temperaturabhängigkeit zurückgeführt werden. Diese Beobachtung zusammen mit der Tatsache, dass die B_1R-Expression unter pathophysiologischen Bedingungen besonders induziert wird, deutet darauf hin, dass der B_1R in Kombination mit Fieber eine verstärkte Wirkung im Organismus haben könnte. Ob diese Heilungs-fördernd oder -abträglich wirkt, müsste noch genauer untersucht werden.

Neben dem Einfluss der Temperatur wird die Signalübertragung der GPCRs durch die jeweiligen Rezeptorkonformationen und die sich daraus ergebenden Funktionsunterschiede bestimmt. Die Familie A der GPCRs wird durch einige hoch konservierte Strukturmerkmale wie die E/DRY-Sequenz mit R3.50 in der dritten Transmembrandomäne (TM) oder die NPXXY-Sequenz am Ende der siebten TM charakterisiert. Publizierte Ergebnisse deuten darauf hin, dass bovines Rhodopsin durch eine Salzbrücke zwischen $R3.50^{135}$ (TM3) und $E6.30^{247}$ (TM6), auch „ionic lock“ genannt, im inaktiven Zustand gehalten wird. Der B_2R ist einer der wenigen Peptid-GPCRs, der ein Glutamat an Position 6.30 ($E6.30^{238}$) trägt, und eignete sich daher zur Untersuchung der Anwesenheit und Funktion eines möglichen „ionic lock“ auch in „nicht-Rhodopsin“-GPCRs. Für alle bisher entsprechend untersuchten GPCRs ist bekannt, dass R3.50 für eine effiziente G-Protein-Aktivierung unabdingbar ist (selbiges wurde in der vorliegenden Arbeit auch für den B_2R bestätigt). Die funktionelle Analyse eines „ionic lock“ anhand einer R3.50 Mutation und G-Protein-abhängiger Kaskaden ist deshalb nicht möglich. Die Rolle eines „ionic lock“ im Hinblick auf G-Protein-unabhängige Mechanismen wie die Rezeptorinternalisierung, einem wichtigen Regulationsschritt für die meisten GPCRs, wurde bisher jedoch noch nicht untersucht.
In der vorliegenden Arbeit wurde erstmals gezeigt, dass die Rezeptorendozytose durch Mutation von $R3.50^{128}$ zu Alanin ($R3.50^{128}A$), im Gegensatz zur G-Protein-Aktivierung, nicht

zum Erliegen kommt. Die mutierten Rezeptorkonstrukte wiesen sogar ein konstitutives Internalisierungsverhalten auf. Dies verwies auf unterschiedliche Funktionen dieser Aminosäure bei der G-Protein-vermittelten Signaltransduktion und bei der Rezeptorinternalisierung.

Ein Aufbrechen des möglichen „ionic lock“ durch Mutation von $E6.30^{238}$ zu Alanin oder Arginin resultierte ebenfalls in konstitutiv internalisierenden Rezeptorkonstrukten. Im Gegensatz zur Endozytose zeigten diese Mutanten zwar keine konstitutive Signalübertragung, wurden aber auch durch prinzipiell als Antagonisten klassifizierte Verbindungen effizient aktiviert. Diese Ergebnisse legen einen mehrstufigen Aktivierungsprozess nahe, dessen Stufen sich durch verschiedene Rezeptorkonformationen mit unterschiedlichen Interaktionsmöglichkeiten für die G-Protein-Rekrutierung/Aktivierung oder mit der Internalisierungsmaschinerie [GPCR-Kinasen (GRKs), Arrestine] auszeichnen. Der wechselseitige Austausch der beiden hoch konservierten Aminosäuren $R3.50^{128}$ und $E6.30^{238}$ ermöglichte wahrscheinlich die Bildung eines inversen „ionic lock“, wodurch normales B_2R-Verhalten wieder hergestellt wurde.

Diese Arbeit zeigt somit erstmals, dass ein Aufbrechen eines möglichen „ionic lock“ in einem Peptidrezeptor unterschiedliche Auswirkungen für die Prozesse der G-Protein-Aktivierung und der Rezeptorendozytose haben kann. Dadurch wird die Annahme bestärkt, dass es bei einem GPCR mehrere aktive Konformationen geben kann, die unterschiedliche Affinitäten gegenüber regulatorischen Proteinen (GRKs, Arrestinen) oder Effektoren (G-Proteinen, Arrestinen) aufweisen und dadurch differenziert zelluläre Signale auslösen können. Die Aufklärung der unterschiedlichen Aktivierungsmechanismen von GPCRs in Kombination mit der Herstellung von Verbindungen z.B. sogenannten „small molecule compounds“, die bestimmte Rezeptorkonformationen mit ihren signalspezifischen Eigenschaften stabilisieren können, wäre möglicherweise für die Entwicklung von Agonisten oder Antagonisten, die nur ganz bestimmte Signalwege modulieren und so eine optimierte therapeutische Anwendung erlauben, hilfreich.

2 Summary

The family A of G protein-coupled receptors (GPCRs) represents the largest and most diverse family of transmembrane receptors. Its members play a pivotal role in a variety of patho(physiological) processes. Upon agonist binding, GPCRs activate heterotrimeric G proteins as well as G protein-independent signaling pathways. Once activated, the different G protein subunits (Gα-GTP and βγ) dissociate from the receptor and stimulate appropriate signaling cascades for example via phospholipase A and Cβ. To prevent dysregulation of cellular processes, GPCRs are strictly regulated by processes determining their signaling ability and their cell surface availability.

The bradykinin B_1 and B_2 receptors (B_1R, B_2R) belong to the family A of GPCRs (rhodopsin-like GPCRs) and are activated by the pro-inflammatory peptides desArg^9-bradykinin/desArg^{10}-kallidin (DABK/DAK) or bradykinin (BK)/kallidin, respectively. Contrary to the constitutively expressed B_2R, which upon stimulation gets rapidly desensitized and internalized, B_1R expression is induced by cytokines almost exclusively under pathophysiological conditions. Following stimulation, the B_1R remains at the cell surface, rather than undergoing internalization. Both receptors couple to $G\alpha_{q/11}$ as well as to $G\alpha_i$ activating almost identical signaling pathways [phospholipase Cβ (PLCβ), mitogen activated protein kinases (MAPK)]. Due to their contrary regulatory patterns, especially with regard to their internalization behaviour, bradykinin receptors represent an interesting model system to analyze GPCR regulation and its impact on their signal transduction.

Both bradykinin receptors are involved in inflammatory events triggering the release of pro-inflammatoy cytokines and the recruitment of immune cells. During inflammation massive cytokine release, such as Interleukin-1β (IL-1β), is induced promoting *de novo* synthesis of B_1Rs. Besides the induction of B_1R expression, pro-inflammatory cytokines like IL-1β lead to an increase of body temperature (fever), a frequent side-effect of inflammation. In spite of the established relation between inflammation and elevated body temperature, knowledge about the influence of a temperature rise on membrane receptors and their signaling activity on a cellular level was very limited.

To our knowledge, the present study is the first to have considered elevated temperature as an important regulatory component for GPCR signaling. Using bradykinin receptors it was shown that temperature can crucially regulate GPCR signal strength. However, differentiation between a temperature dependence of the signaling pathway itself and a receptor-specific temperature sensitivity was required. ERK1/2 activation for one, was significantly increased under pathophysiologically elevated temperatures (41°C; normal body temperature: 37°C)

upon stimulation of both kinin receptors (receptor-independently). However, only a B_1R-specific temperature dependence was responsible for elevated PLCβ-mediated signaling at 41°C. These findings together with the fact that B_1R expression is induced under pathophysiological conditions suggest that the B_1R in combination with elevated temperature might have special effects on the whole organism. Whether these are beneficial or detrimental to the healing process has to be further investigated.

Besides the influence of temperature, GPCR signaling is determined by receptor conformations and resulting functional differences. Family A GPCRs are characterized by a few highly conserved motifs such as the E/DRY sequence with R3.50 in transmembrane (TM) 3 or the NPXXY domain at the end of TM 7. Published data have shown that bovine rhodopsin is maintained inactive by a salt bridge between $R3.50^{135}$ and $E6.30^{247}$ in TM 6, also known as ionic lock. Since the B_2R is one of the few peptide GPCRs carrying a glutamate at position 6.30 ($E6.30^{238}$) it was suitable for the analysis of the presence and function of a possible ionic lock also in nonrhodopsin GPCRs. For all previously analyzed GPCRs it has been shown that R3.50 is indispensable for efficient G protein activation (in the present study also confirmed for the B_2R). Thus, the functional analysis of an ionic lock via mutation of R3.50 and G protein-dependent mechanisms is not possible. Until now, the role of an ionic lock considering G protein-independent mechanisms such as receptor internalization, an important regulatory process for many GPCRs, has not been investigated yet.

Herein it was demonstrated for the first time that, in contrast to G protein activation, substitution of $R3.50^{128}$ with alanine ($R3.50^{128}A$) had no negative impact on B_2R internalization. These G protein activation-incompetent constructs displayed constitutive internalization indicating a dual role of this residue in G protein-mediated signal transduction and in receptor internalization.

Disruption of the putative ionic lock by mutating $E6.30^{238}$ to alanine or arginine similarly resulted in constitutive internalization. Contrary to their endocytosis behaviour, these mutants displayed no constitutive signal transduction, but were efficiently activated even by compounds classified as antagonists. These results suggest a multistep process of GPCR activation characterized by various receptor conformations with different propensities for interaction either for G protein recruitment/activation or with the endocytosis machinery [GPCR kinases (GRKs), arrestins]. Mutual swapping of the two highly conserved residues $R3.50^{128}$ and $E6.30^{238}$ allowed the formation of an „inverse“ ionic lock and thereby reconstituted normal B_2R behaviour.

These findings show for the first time that disruption of a possible ionic lock in a peptide receptor can have different effects with regard to G protein activation and receptor endocytosis. Therefore, the existence of multiple active GPCR conformations is feasible, each of them displaying different affinities for regulatory proteins (GRKs, arrestins) or effectors (G proteins, arrestins), thereby inducing different cellular effects. Deeper insight into the diversity of GPCR activation mechanisms combined with the development of so called “small molecule compounds“ that stabilize specific receptor conformations and their signal-specific properties might help to develop agonists or antagonists, which differentially affect the various signaling pathways of interest, and thus allow an optimized therapeutic intervention.

3 Die Familie der G-Protein-gekoppelten Rezeptoren

G-Protein-gekoppelte Rezeptoren (GPCRs) bestehen aus 7 α-helikalen Transmembrandomänen (TM) verbunden durch alternierende extra- und intrazelluläre Schleifen und bilden mit 800-1000 Mitgliedern die größte Familie der Membranrezeptoren (Palczewski et al., 2000). Stimuliert durch Hormone, Neurotransmitter, Lipide, Peptide, Ionen und sensorische Stimuli, sind sie an einer Vielzahl physiologischer und pathophysiologischer Prozesse beteiligt, zu denen unter anderem die Herzfunktion, die Neurotransmission, die Blutdruckregulation, die Embryogenese, oder die Wahrnehmung von Licht, Geruch und Geschmack zu zählen sind (Shenoy and Lefkowitz, 2005). Schätzungsweise 25-50% aller auf dem Markt befindlichen Arzneimittel wirken direkt oder indirekt über GPCRs, womit diese Rezeptorfamilie den wichtigsten pharmakologischen Ansatzpunkt für die Therapie von Krankheiten darstellt.

3.1 Signalvermittlung und Regulation von GPCRs im Allgemeinen

Die Aktivierung von GPCRs durch Bindung entsprechender Liganden an extrazelluläre Bereiche und/oder Transmembranregionen führt in selbigen zu Konformationsänderungen in den Transmembran- und intrazellulären Domänen, die dadurch für eine Interaktion mit heterotrimeren G-Proteinen zugänglich werden. Aktive GPCRs wirken als Guaninnukleotid-austauschfaktoren und katalysieren somit den Austausch von GDP zu GTP an den entsprechenden Gα-Untereinheiten. Die dadurch aktivierten G-Protein-Untereinheiten (Gα-GTP und βγ) dissoziieren vom Rezeptor und fördern durch Interaktion mit diversen Effektorproteinen z.B. Adenylatzyklasen, Phospholipasen oder Ionenkanälen, die Freisetzung von sekundären Botenstoffen wie Calcium und Phosphoinositiden (Birnbaumer, 2007; Ritter and Hall, 2009). In Säugetieren existieren 16 Gα-, 5 Gβ- und 12 Gγ-Untereinheiten (Shukla et al., 2011). Die Gα-Untereinheiten werden nach Sequenzähnlichkeit in 4 Klassen ($G\alpha_s$,$G\alpha_{q/11}$,$G\alpha_i$,$G\alpha_{12/13}$) unterteilt, die mit unterschiedlichen Effektorproteinen interagieren und diverse Signalkaskaden stimulieren können (Wess, 1998) (Abb. 1). So modulieren beispielsweise $G\alpha_s$- und $G\alpha_i$-Untereinheiten, aber auch βγ-Dimere, die Adenylatzyklasen (AC) und kontrollieren damit die Menge an intrazellulärem zyklischen Adenosinmonophosphat (cAMP). $G\alpha_{q/11}$ dagegen aktiviert Phospholipase Cβ (PLCβ) und deren nachgeschaltete Signalwege (Ritter and Hall, 2009) (Abb. 1).

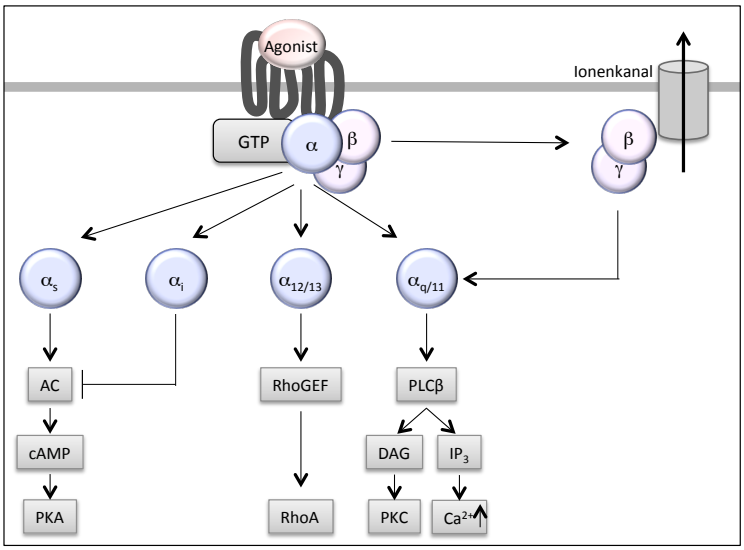


Abb. 1. Vereinfachte Darstellung G-Protein-vermittelter Signalübertragung. Durch Bindung eines Agonisten kommt es zur Konformationsänderung des G-Protein-gekoppelten Rezeptors (GPCR) und dadurch zur Aktivierung eines oder mehrerer heterotrimerer G-Proteine, die verschiedene Effektoren stimulieren können. Diese vermitteln weitere zelluläre Signale. AC: Adenylatzyklase; cAMP: zyklisches Adenosin-monophosphat; PKA: Proteinkinase A; RhoGEF: Rho Guaninnukleotid-austauschfaktor; PLCβ: Phospholipase Cβ; DAG: Diacylglycerol; IP_3: Inositol-1,4,5-triphosphat; PKC: Proteinkinase C; Ca^{2+}: Calcium (modifiziert nach Ritter and Hall, 2009).

Neben der hohen Anzahl an Kombinationsmöglichkeiten der G-Protein-Untereinheiten und -Subtypen, wird die biochemische Komplexität GPCR-induzierter Signalübertragung durch unterschiedliche Expressionsmuster der Rezeptoren, der regulatorischen Proteine und ihrer Effektorenzyme erreicht. Weiterhin kann ein aktiver GPCR oft auch unterschiedliche G-Proteine aktivieren. Um eine Fehlregulation zellulärer Prozesse durch eine anhaltende Signaltransduktion bzw. „Überstimulation“ zu vermeiden, unterliegen diese Rezeptoren strengen Regulationsmechanismen. Einen wesentlichen Beitrag zum Abschalten G-Protein-abhängiger Signalkaskaden leisten die zwei Proteinfamilien der GPCR-Kinasen (GRKs) (Pitcher et al., 1998) und der Arrestine (Shenoy and Lefkowitz, 2003), die fast ausschließlich mit Liganden-aktivierten GPCRs interagieren. Die Enzymfamilie der GRKs besteht aus 7 Mitgliedern, wobei die einzelnen Subtypen unterschiedliche gewebespezifische Expressionsniveaus zeigen und bestimmte GPCRs als Substrate bevorzugen können. GRK1 und 7 werden in Stäbchen und Zapfen der Netzhaut exprimiert, GRK4 ist hauptsächlich in Kleinhirn, Hoden und Nieren zu finden. GRK2, 3, 5 und 6 zeigen dagegen ein nahezu ubiquitäres Expressionsmuster. GRKs phosphorylieren aktivierte GPCRs an Serinen und Threoninen überwiegend in deren C-Terminus, woraus anschließend die Rekrutierung von Arrestinen resultiert (Drake et al., 2006). Diese multifunktionellen Adapterproteine (Arrestin1-4), von denen nur Arrestin2 (β-Arrestin1) und 3 (β-Arrestin2) ubiquitär auch außerhalb der Sehzellen exprimiert werden, unterbrechen sterisch die weitere Kopplung der aktivierten GPCRs an ihre G-Proteine (Desensitisierung) und vermitteln anschließend häufig die Rezeptorendozytose über sogenannte „clathrin-coated pits“ (Reiter and Lefkowitz, 2006). Nach ihrer Internalisierung werden GPCRs entweder lysosomal degradiert oder

dephosphoryliert und zurück an die Zelloberfläche transportiert (Recycling) (Ritter and Hall, 2009) (Abb. 2).

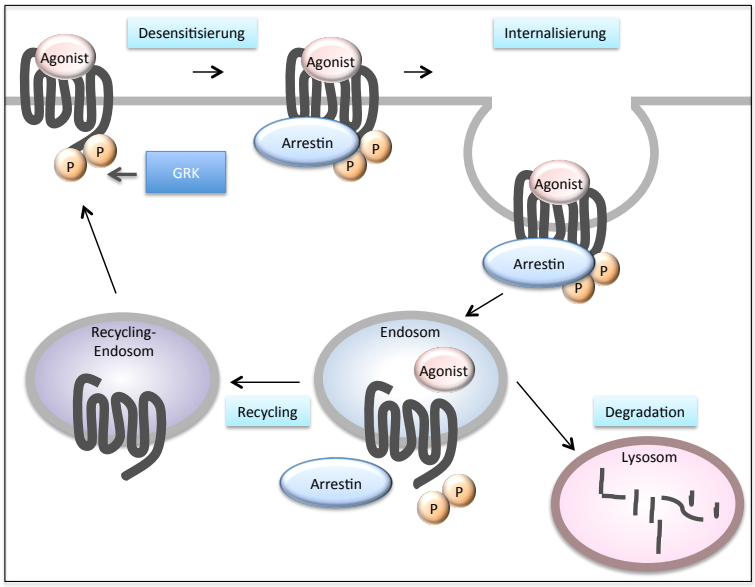


Abb. 2. Klassische Regulationsmechanismen von G-Protein-gekoppelten Rezeptoren (GPCRs). Phosphorylierung von GPCRs durch GPCR-Kinasen (GRKs) führt zur Rekrutierung von Arrestinen. Diese verhindern die weitere Anlagerung von G-Proteinen (Desensitisierung) und vermitteln oft die Internalisierung der GPCRs. Der internalisierte Rezeptor wird entweder in Lysosomen degradiert, oder über Recycling-Endosomen zurück an die Zelloberfläche transportiert (Resensitisierung).

Die in den Säugerzellen existierenden 4 ubiquitär vorkommenden GRK- und die zwei β-Arrestin-Subtypen sind wahrscheinlich mehr oder weniger für die Regulation aller 800-1000 GPCRs verantwortlich (Shukla et al., 2011). Inwieweit sich die Regulationsmechanismen der einzelnen GPCRs unterscheiden oder gleichen, bzw. ob es bei den Rezeptoren gemeinsame Strukturmerkmale gibt, die den jeweiligen Regulationsweg spezifizieren, ist noch weitgehend ungeklärt.

3.2 Molekulare Regulationsmechanismen und Signalübertragung von GPCRs am Beispiel der Bradykininrezeptoren

In Vertebraten unterscheidet man aufgrund ihrer Strukturähnlichkeit 5 GPCR-Familien: die Rhodopsin- (Familie A), die Secretin- (Familie B), die Glutamat- (Familie C), die Adhäsions- und Frizzled/Taste2-Rezeptoren (Fredriksson et al., 2003). Die Bradykininrezeptoren B_1 und B_2 (B_1R und B_2R), wichtige Mediatoren des Kallikrein-Kinin-Systems (KKS), gehören zur Familie A, der größten und vielfältigsten aller GPCR-Familien (Leeb-Lundberg et al., 2005). Die pro-inflammatorischen Peptide Bradykinin (BK: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) und Kallidin (Lys-BK), die Agonisten des B_2R, werden bei Entzündungsprozessen aus den Kininogenen, die in der Leber synthetisiert und in den Blutkreislauf sezerniert werden, durch Plasma- und Gewebskallikreine (Serinproteasen) freigesetzt. Die Entfernung des jeweiligen C-terminalen Arginins durch Carboxypeptidasen generiert die Agonisten für den B_1R,

desArg^{9}-BK/desArg^{10}-Kallidin (DABK/DAK) (Abb. 3), für die der B_2R fast keine Affinität mehr zeigt.

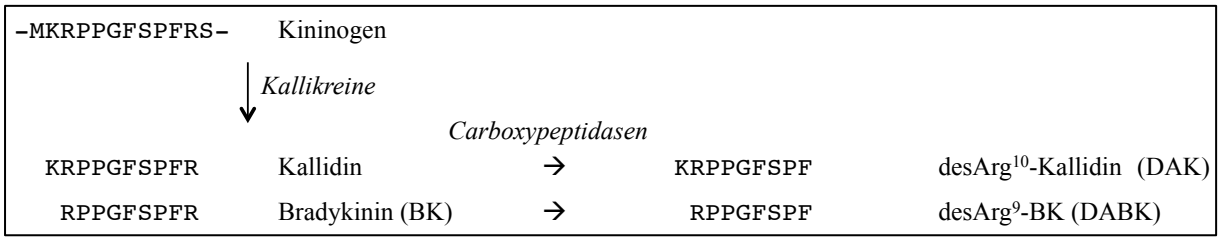


Abb. 3. Bildung der pro-inflammatorischen Peptidagonisten der Bradykininrezeptoren.

Beide Bradykininrezeptoren sind an wichtigen physiologischen und pathophysiologischen Prozessen beteiligt, wie beispielsweise der Vasodilatation, der Erhöhung der Gefäßpermeabilität, der Schmerzentstehung und der Ödembildung. Ihnen wird vor allem bei kardiovaskulären Erkrankungen und einigen inflammatorischen Konditionen (Asthma, Rhinitis) eine Funktion zugeschrieben (Leeb-Lundberg et al., 2005).

Die meisten physiologischen Kinineffekte werden durch Aktivierung des B_2R ausgelöst. Dieser wird konstitutiv in fast allen Zellarten (z.B. Endothelzellen, glatten Muskelzellen, Fibroblasten, Nervenzellen) exprimiert und ist, wenn kein Ligand gebunden hat, hauptsächlich an der Zelloberfläche lokalisiert. Der Rezeptor wird, nach Bindung von BK und Signalvermittlung in die Zelle, an fünf C-terminalen Serinen und Threoninen in erster Linie von GRK2/3 phosphoryliert. Daraufhin kommt es zu einer schnellen Desensitisierung durch Assoziation mit β-Arrestin1 und 2, welche die weitere Anlagerung und Aktivierung von G-Proteinen verhindert. Die β-Arrestin-Interaktion führt außerdem zur Internalisierung des Rezeptors über „clathrin-coated pits“ (Blaukat et al., 2001; Leeb-Lundberg et al., 2005). Der Großteil der B_2R wird nach der Endozytose über Recycling-Endosomen zurück an die Zelloberfläche transportiert (Recycling). Ein geringer Anteil wird degradiert (Enquist et al., 2007).

Im Gegensatz zum B_2R erfolgt eine B_1R-Expression erst nach Ausschüttung pro-inflammatorischer Zytokine wie Interleukin-1β (IL-1β) unter pathophysiologischen Bedingungen. Der B_1R wird nicht phosphoryliert oder desensitisiert (Alhenc-Gelas et al., 2011), sondern ist in manchen Zellarten nach Bindung seiner Agonisten DAK oder DABK sogar verstärkt an der Zelloberfläche zu finden (Phagoo et al., 2001).

3.2.1 Temperaturabhängigkeit der Signaltransduktion von B_1R und B_2R

Kinine, die Agonisten der Bradykininrezeptoren, werden vermehrt im Bereich von Entzündungsherden ausgeschüttet und führen dann zur Aktivierung der B_1 und B_2 Rezeptoren (Leeb-Lundberg et al., 2005). Die Hauptsymptome von Entzündungsreaktionen sind Schmerz, Rötung, Schwellung, eingeschränkte Funktion und Erwärmung (Elliott et al., 1960). IL-1β, welches die B_1R-Expression unter pathophysiologischen Bedingungen induziert, ist außerdem maßgeblich an der Fieberentstehung während der Immunabwehr beteiligt (Jansky et al., 1995; Phagoo et al., 2001; Roth and De Souza, 2001). Erste Hinweise - wie ein stark vermindertes Calcium-Signal nach B_1R-Stimulation bei 25°C im Vergleich zu 37°C - sowie der Zusammenhang zwischen IL-1β mit steigender Körpertemperatur und IL-1β-vermittelter B_1R-Induktion, ließen eine Temperaturabhängigkeit dieses Rezeptors vermuten. Erstaunlicherweise wurde unserer Kenntnis nach bisher nicht untersucht, inwieweit eine erhöhte Temperatur als Begleiterscheinung fast aller entzündlicher Vorgänge die Signalaktivität von Membranrezeptoren, die am Entzündungsgeschehen beteiligt sind, beeinflusst. Mit ihren konträren Regulationsmustern, vor allem was die Internalisierung angeht, stellen die Bradykininrezeptoren prinzipiell ein interessantes System zur Analyse regulatorischer Mechanismen in GPCRs dar. Im Rahmen dieser Doktorarbeit wurde auf zellulärer Ebene erstmals auch der Einfluss pathophysiologisch erhöhter Temperatur (41°C) gegenüber normaler Körptertemperatur (37°C) auf die Signalvermittlung von GPCRs am Modell der B_1 und B_2 Rezeptoren analysiert (Publikation A).

Beide Rezeptoren aktivieren nach Ligandenbindung Signalkaskaden über die G-Protein-Subtypen $G\alpha_{q/11}$ und $G\alpha_i$. Aktives $G\alpha_{q/11}$ stimuliert Phospholipase Cβ (PLCβ), welche durch Hydrolyse von Phosphatidylinositol 4,5-bisphosphat die sekundären Botenstoffe Inositol 1,4,5-triphosphat (IP_3) und Diacylglycerol (DAG) generiert (Abb. 2). IP_3 induziert die Freisetzung von Calcium (Ca^{2+}) aus intrazellulären Speichern, wodurch unter anderem der Transkriptionsfaktor „nuclear factor of activated T cells“ (NFAT) und dessen Zielgene, wie Autotaxin oder Cyclooxygenase 2, aktiviert werden (Müller and Rao, 2010).

Der B_2R stimuliert über $G\alpha_{q/11}$ und $G\alpha_i$ auch die „extracellular signal-regulated kinase“ (ERK1/2)-Kaskade. Die Aktivierung von ERK1/2, einer zentralen Effektorkinase der „mitogen-activated protein kinases“ (MAPK), geht mit ihrer Phosphorylierung einher (Blaukat et al., 2000). Wir konnten auch für den B_1R eine duale G-Protein-Kopplung zur Stimulation dieses Signalwegs zeigen (Publikation A). Zu den zahlreichen Substraten von ERK1/2 zählt neben c-Fos und Elk-1 auch der Transkriptionsfaktor „activator protein 1“ (AP-1), worüber langfristig wichtige Kontrollpunkte des Zellzyklus, wie Cyclin D1, reguliert

werden (Yordy and Muise-Helmericks, 2000; Pruitt and Der, 2001). Im Hinblick auf eine Temperaturabhängigkeit von Membranrezeptoren wurden im Rahmen dieser Arbeit sowohl kurzfristige Signale [Phosphatidylinositol (PI)-Hydrolyse und ERK1/2-Aktivierung], als auch längerfristige Effekte der Kininrezeptoren (NFAT- und AP-1-Reportergenaktivierung) untersucht.

Die Forschungsergebnisse unserer Studie belegen die Notwendigkeit, zwischen einer Temperaturabhängigkeit von Signalwegen selbst, im Gegensatz zu einer spezifischen Temperaturempfindlichkeit des untersuchten GPCRs zu unterscheiden. Eine pathophysiologische Temperaturerhöhung um nur 4°C, von 37°C auf 41°C, verdoppelte die ERK1/2-Phosphorylierung, unabhängig davon, ob sie durch den B_1R oder den B_2R aktiviert wurde. Ein signifikanter Anstieg der AP-1-Reportergenaktivierung wurde ebenso für B_1R- wie für B_2R-vermittelte Signale beobachtet. Damit ist die temperaturinduzierte Erhöhung der ERK1/2-Signalaktivität ein Mechanismus, der wahrscheinlich auf Proteinkinase C (PKC)-aktivierende GPCRs im Allgemeinen zutrifft. Im Gegensatz dazu wurde ein temperaturabhängiger Anstieg der PI-Hydrolyse sowie auch der Aktivierung des NFAT-Reportergens nur bei B_1R-Stimulation, also spezifisch für diesen GPCR, festgestellt.

Eine Aktivierung des B_2R führt vor allem zu einer transienten Signalvermittlung (schnelle Desensitisierung) während der akuten Entzündungsphase. Im Gegensatz dazu sind B_1R-induzierte Signalwege von längerer Dauer (keine Desensitisierung) und können daher in der chronischen Phase der Immunantwort inflammatorische Reaktionen amplifizieren (Leeb-Lundberg et al., 2005). Unsere Ergebnisse zusammen mit den unterschiedlichen Regulations- und Expressionsmustern von B_1R (induzierbar) und B_2R (konstitutiv) deuten darauf hin, dass bei Entzündungsvorgängen in Verbindung mit Fieber B_1R-vermittelte Signalwege im Vordergrund stehen. Wodurch die starke Temperaturabhängigkeit des B_1R zustande kommt, ob z.B. durch eine effizientere G-Protein-Kopplung oder durch z.B. eine Beteiligung temperatursensitiver Ca^{2+}-Kanäle bleibt noch zu klären.

Die in der vorliegenden Arbeit präsentierten Daten weisen darauf hin, dass Informationen über die Temperaturabhängigkeit relevanter Membranrezeptoren und ihrer zellulären Signale von Bedeutung sein könnten, um dies bei der Entscheidung, ob in einem bestimmten Fall Fieber therapeutisch gesenkt werden soll, entsprechend berücksichtigen zu können.

3.2.2 Die Struktur des Rezeptors und ihr Einfluss auf seine Funktion: Der B_2R als Modell

Obwohl knapp die Hälfte aller Arzneimittel auf GPCRs wirken, ist deren Potential in diesem Bereich noch ganz und gar nicht erschöpft, denn immer noch fehlen genauere Kenntnisse über Unterschiede und Gemeinsamkeiten der regulatorischen Mechanismen bzw. des Aktivierungsprozesses dieser vielfältigen Rezeptorgruppe (Jacoby et al., 2006), die zur Entwicklung von weiteren, spezifischeren Medikamenten beitragen könnten. Abgesehen von dem Einfluss der Temperatur, kann die Stärke und Dauer GPCR-vermittelter Signale durch Regulationsmechanismen wie die Desensitisierung, die Internalisierung, die Resensitisierung und die Degradation bestimmt werden (siehe Abb. 2). Liganden-induzierte Endozytose beispielsweise verringert die Rezeptoranzahl an der Zelloberfläche, wodurch eine übermäßige Stimulation verhindert wird. Derartige Vorgänge werden wahrscheinlich durch spezifische Strukturmerkmale und Bindungsdomänen für GRKs oder Arrestine der jeweiligen Rezeptoren reguliert. Die Familie A der GPCRs wird durch wenige hoch konservierte Domänen charakterisiert (Palczewski et al., 2000). Solch konservierte Bereiche sind zum einen die E/DRY-Sequenz in der dritten Transmembrandomäne (TM) und zum anderen das NPXXY-Motiv am Ende der siebten TM (Abb. 4). Um die einzelnen Aminosäuren von GPCRs in Bezug auf ihre Position in den entsprechenden Rezeptoren vergleichen zu können, wird im Folgenden die Nomenklatur nach Ballesteros und Weinstein verwendet (Ballesteros et al., 1998): Die Aminosäuren der Transmembranbereiche werden mit je zwei Nummern angegeben, wobei die erste Zahl die TM (1-7) bezeichnet und die zweite Nummer für die Position relativ zu der am höchsten konservierten Aminosäure der TM steht, der die Nummer 50 zugeordnet wird. In Richtung N-Terminus wird abwärts, in Richtung C-Terminus aufwärts gezählt.

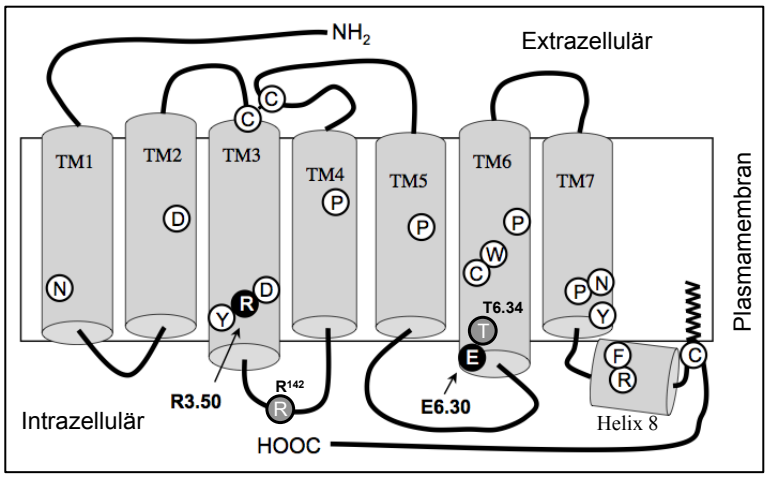


Abb. 4. Schematische Darstellung der Struktur des Bradykininrezeptors B_2 (B_2R). Hoch konservierte Aminosäuren sind als weiße Kreise mit dem Einbuchstabencode der Amino-säuren abgebildet. Die beiden am „ionic lock" beteiligten Aminosäuren sind als schwarze Kreise mit weißer Schrift dargestellt. Grau gefüllte Kreise zeigen die Aminosäuren, die die Stabilität des „ionic lock" mitbestimmen [modifiziert nach Leschner et al., 2012 (Publikation B)].

Anhand verschiedener Kristallstrukturen konnte der Aktivierungsmechanismus von Rhodopsin ziemlich detailliert aufgeklärt werden. Über den Ablauf der Rezeptoraktivierung in anderen GPCRs der Familie A wie beispielsweise den Peptidrezeptoren ist hingegen deutlich weniger bekannt.

In inaktivem Rhodopsin wird die dritte TM mit der sechsten über eine Salzbrücke zwischen Arginin 135 ($R3.50^{135}$), Teil des E/DRY Motivs, und Glutamat 247 ($E6.30^{247}$) in der sechsten TM des Rezeptors (Palczewski et al., 2000), verbunden. Diese Interaktion hält den Rezeptor im inaktiven Zustand und wird daher auch als „ionic lock" bezeichnet (Teller et al., 2001; Okada et al., 2004; Vogel et al., 2008). R3.50 ist die meist konservierte Aminosäure in GPCRs der Familie A (96%) (Mirzadegan et al., 2003), wohingegen eine saure Aminosäure an Position 6.30 zwar in beinahe allen Aminrezeptoren, jedoch nur in weniger als 7% der Peptidrezeptoren zu finden ist (Springael et al., 2007). Der B_2R ist einer der seltenen Peptidrezeptoren mit einem Glutamat an Position 6.30. Es besteht also, im Gegensatz zu den meisten Peptidrezeptoren, potentiell die Möglichkeit zur Bildung eines „ionic lock". Damit könnte sich der Aktivierungsprozess des B_2R von dem anderer Peptidrezeptoren abgrenzen und möglicherweise interessante Ansatzpunkte für die Entwicklung spezifischer Agonisten oder Antagonisten liefern.

3.2.2.1 Die DRY-Sequenz und ihre Funktion für die G-Protein-Aktivierung

Bisherige Untersuchungen zur Funktion eines „ionic lock" in GPCRs konzentrierten sich hauptsächlich auf R3.50. Dieses Arginin ist für eine effiziente G-Protein-Aktivierung in den meisten GPCRs unentbehrlich (Rovati et al., 2007). Auch der B_2R verliert durch Substitution von $R3.50^{128}$ mit anderen Aminosäuren komplett die Fähigkeit zur Liganden-stimulierten PI-Hydrolyse und ERK1/2-Phosphorylierung (Publikation B). Im Gegensatz zu anderen Membranrezeptoren wie z.B. dem Angiotensin II Typ 1 Rezeptor, bei dem die ERK1/2-Phosphorylierung auch über β-Arrestin2 vermittelt werden soll (Wei et al., 2003), läuft die MAPK-Signaltransduktion beim B_2R, zumindest in HEK293 Zellen, ausschließlich über die Aktivierung von $G\alpha_{q/11}$ und $G\alpha_i$ (Publikation A und B).

Um die Funktion eines möglichen „ionic lock" zwischen R3.50 und E6.30 in einem „nicht-Rhodopsin"-GPCR zu untersuchen, analysierten wir verschiedene Rezeptormutanten, in denen entweder $R3.50^{128}$, $E6.30^{238}$ oder beide Aminosäuren mutiert wurden. Bei der Charakterisierung dieser Mutanten wurde der Fokus nicht nur auf deren Signalübertragung gelegt, sondern erstmals auch auf G-Protein-unabhängige Mechanismen wie die

Internalisierung (Shenoy and Lefkowitz, 2005; Shukla et al., 2011), die maßgeblich an der Regulation der GPCR-induzierten Signalvermittlung beteiligt ist (Publikation B).

3.2.2.2 Das „ionic lock“ im B_2R

Ein erstelltes Strukturmodell des B_2R, das auf der Struktur von bovinem Rhodopsin (PDB: 1U19) basierte, zeigte, dass die Bildung eines „ionic lock“ im B_2R zwischen $R3.50^{128}$ und $E6.30^{238}$ anzunehmen ist. In unserem Modell wird das „ionic lock“ durch ein zusätzliches Netzwerk der Seitenketten von $D3.49^{127}$, $T6.34^{242}$, sowie $A6.33^{241}$ unterstützt (Publikation B). Obwohl eine Mutation von $R3.50^{128}$ zu einem neutralen Alanin im B_2R jegliche G-Protein-abhängige Signalvermittlung inhibiert, wird dadurch weder seine Phosphorylierung noch die Rezeptorinternalisierung verhindert. Diese Ergebnisse sprechen für eine weiterhin mögliche Regulation dieses Rezeptorkonstrukts durch GRKs und β-Arrestine. Bisherigen Annahmen zur Folge assoziieren GRK2/3 nach Agonistenbindung und G-Protein-Aktivierung über ihre Pleckstrin-Homologie-Domäne mit frei gewordenen βγ-Untereinheiten und phosphorylieren den Rezeptor an C-terminalen Serinen und Threoninen. Es kommt zur Assoziation von β-Arrestinen, die eine weitere G-Protein-Anlagerung sterisch verhindern (Desensitisierung) (Willets et al., 2003). Mittlerweile gibt es allerdings Hinweise darauf, dass GRKs auch direkt, ohne vorherige Rekrutierung durch βγ-Untereinheiten, mit dem Rezeptor und $G\alpha_{q/11}$ interagieren können (Lodowski et al., 2003; Willets et al., 2003). Auch für den B_2R wurde von unserer Arbeitgrupppe kürzlich die Helix 8 (Abb. 4) als Vermittlerin der GRK-Interaktion identifiziert (Feierler et al., 2011). Die $R3.50^{128}$A-Mutation dieses Rezeptors könnte folglich eine Konformation induzieren, die direkt mit GRK2/3 interagiert und so die Phosphorylierung des Konstrukts und seine Internalisierung einleitet.

Eine Mutation von $R3.50^{128}$ bzw. $E6.30^{238}$ oder beider Aminosäuren zu Alanin, führte zu erhöhter basaler Phosphorylierung sowie zu konstitutiver Endozytose dieser B_2R-Konstrukte. Ein gegenseitiger Austausch von $R3.50^{128}$ und $E6.30^{238}$, der die Bildung eines inversen „ionic lock“ ermöglichen sollte, rekonstituierte normales Verhalten des Rezeptors. Diese Fakten deuten auf die Existenz eines „ionic lock“ im inaktiven B_2R hin, das in seiner Funktion durch ein Netzwerk der umgebenden Aminosäuren, in dem sowohl $R3.50^{128}$, als auch $E6.30^{238}$ weitere Interaktionspartner haben, unterstützt wird.

3.2.2.3 Der Aktivierungsmechanismus von GPCRs – Ein mehrstufiger Prozess

In den letzten Jahren wurden die Kristallstrukturen einiger GPCRs publiziert. Diese gaben erste Hinweise auf einen mehrstufigen Aktivierungsmechanismus von Membranrezeptoren. Sie zeigen eine Reihe von Konformationen, die möglicherweise in Abhängigkeit vom jeweiligen Liganden unterschiedliche Effekte auslösen können (Salon et al., 2011).

Im Fall des B_2R generierte die Substitution von $E6.30^{238}$ durch Alanin oder Arginin Rezeptorkonstrukte, die nicht nur nach Stimulation mit dem Agonisten BK starke PI-Hydrolyse und ERK1/2–Aktivierung auslösten, sondern auch nach Bindung von prinzipiell als Antagonisten eingestuften Verbindungen effizient die G-Protein-Aktivierung vermittelten (Publikation B). Allerdings zeigten diese mutierten Rezeptoren keine basal erhöhte G-Protein-Stimulation und nahmen demnach eine hoch sensitive, leicht aktivierbare, jedoch nicht konstitutiv aktive Konformation im Hinblick auf die G-Protein-Kopplung ein. Da ein mögliches Aufbrechen des „ionic lock“ durch Mutation von E6.30 Rezeptorkonformationen induzierte, die leicht (auch durch Antagonisten) aktivierbar waren, jedoch noch nicht konstitutiv G-Proteine aktivierten, scheinen also auch im B_2R verschiedene Aktivitätszustände zu existieren. Ähnliches wurde auch an E6.30-Mutanten des Thromboxan-Prostanoid Rezeptors beobachtet, die eine effizientere Signalübertragung ohne Erhöhung der basalen Rezeptoraktivität aufwiesen (Ambrosio et al., 2010). Dass die diversen Aktivitätszustände des B_2R unterschiedliche Auswirkungen auf nachgeschaltete Wege haben können, wurde in der vorliegenden Arbeit anhand der Untersuchung eines G-Protein-unabhängigen Mechanismus, der Internalisierung, gezeigt. Die $E6.30^{238}$-Mutanten wiesen erhöhte basale Phosphorylierung sowie konstitutive Endozytose auf. Ein Aufbrechen eines potentiellen „ionic lock“ durch Mutation von E6.30 hat also verschiedene Auswirkungen: Zum einen eine konstitutive Rezeptorinternalisierung und zum anderen die Induktion hoch sensitiver, jedoch nicht konstitutiver Konformationen mit Hinblick auf G-Protein-Stimulation.

Zusammenfassend zeigen unsere Ergebnisse, dass auch der B_2R durch ein „ionic lock“ zwischen der dritten und der sechsten TM inaktiv gehalten werden könnte. Ein Aufbrechen dieser Interaktion durch Mutation der beteiligten Aminosäuren, $R3.50^{128}$ bzw. $E6.30^{238}$, hat unterschiedliche Auswirkungen auf die Rezeptorinternalisierung und auf das G-Protein Signal. Das hoch konservierte Arginin an Position 3.50 ist für eine effiziente G-Protein-Aktivierung unabkömmlich. Durch Neutralisierung dieser Aminosäure und/oder Substitution des sauren Glutamats in der sechsten TM ($E6.30^{238}$) durch Alanin oder Arginin, nimmt der Rezeptor eine konstitutiv mit GRKs und Arrestinen, jedoch nicht mit G-Proteinen

interagierende Konformation ein. Diese Resultate weisen auf die Existenz mehrerer Aktivitätszustände von GPCRs hin, die variierende Sensitivitäten gegenüber unterschiedlichen Liganden zeigen. Da sich E6.30^{238}A/R-Konstrukte gleichermaßen durch Agonisten wie durch Antagonisten zur G-Protein-Aktivierung stimulieren lassen, induzieren diese Mutationen wahrscheinlich hoch sensitive, semi-aktive Rezeptorstrukturen.
Diese Arbeit gibt somit erstmals Hinweise auf die Existenz eines „ionic lock" in einem Peptid-GPCR, dessen Aufbrechen verschiedene Aktivitätszustände generieren könnte.

4 Physiologische Relevanz der Arbeit

Die zelluläre GPCR-vermittelte Signalübertragung spielt in einer Reihe von Krankheitsbildern, z.B. in kardiovaskulären oder renalen Erkrankungen, in Autoimmunerkrankungen und in inflammatorischen Prozessen, eine zentrale Rolle (Reiter et al., 2012). Die Vielfältigkeit der Signalübertragung durch GPCRs wird auf mehreren Ebenen reguliert. Zum einen können durch variierende Liganden-induzierte Rezeptorkonformationen unterschiedliche Effektorkaskaden induziert werden. Zum anderen kann die GPCR-vermittelte Signaltransduktion durch äußere Einflüsse wie die Temperatur reguliert werden. Erhöhte Temperatur gehört zu den Kardinalsymptomen inflammatorischer Prozesse. Besonders die spezifische Temperaturabhängigkeit der Signalvermittlung über den B_1R könnte dabei therapeutisch interessant sein. Kürzlich wurde gezeigt, dass der Cholesterinsenker Lovastatin in Ratten, bei denen durch Pilocarpin ein „*Status Epilepticus*" ausgelöst wurde, sowohl bestimmte Zytokinlevel (IL-1β, IL-6), als auch die B_1R-Expression und die Körpertemperatur verringerte (Gouveia et al., 2011). Es wäre möglich, dass die beobachtete neuroprotektive Wirkung von Lovastatin nicht nur auf die geringere Rezeptorexpression, sondern zusätzlich auch auf eine verringerte B_1R-vermittelte Signalübertragung bei der reduzierten Körpertemperatur zurückzuführen ist. Bisher wird eine Modulation der B_1R-Funktion jedoch nicht therapeutisch angewandt.
Der B_2R hingegen wird bei der Behandlung des hereditären Angioödems, welches mit einer lebensbedrohlichen BK-vermittelten Erhöhung der Gefäßpermeabilität einhergeht, mittels Icatibant antagonisiert (Greve et al., 2011).
Die Mittel der Wahl bei Bluthochdruck, sowie bei Herz- und Nierenversagen, sind „Angiotensin-converting-enzyme" (ACE)-Inhibitoren (ACEI) oder Angiotensin-Rezeptor-Blocker (ARB) (Alhenc-Gelas et al., 2011; Regoli et al., 2012). Diese blockieren das Renin-Angiotensin-Aldosteron-System (RAAS), führen aber auch zu einer Akkumulation von BK

und aktivieren dadurch das gegenregulierende, vasodilatorische Kallikrein-Kinin-System (KKS) (Abb. 5).

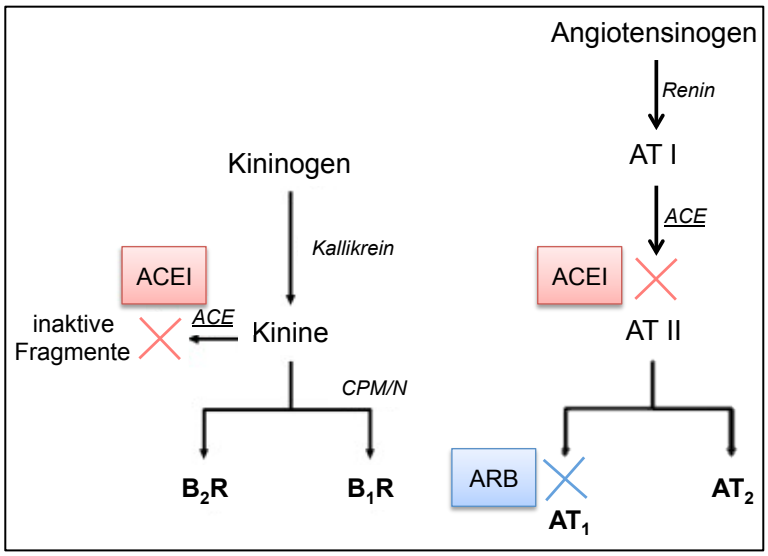


Abb. 5. Zusammenspiel des Renin-Angiotensin-Aldosteron-Systems (RAAS) mit dem Kallikrein-Kinin-System (KKS). Enzymatische Bildung von Angiotensinen und Kininen mit den jeweiligen Angriffspunkten von „Angiotensin-converting-enzyme" (ACE)-Inhibitoren (ACEI) und Angiotensin-Rezeptor-Blockern (ARB). AT I: Angiotensin I; AT II: Angiotensin II; AT_1: Angiotensin II Typ 1-Rezeptor; AT_2: Angiotensin II Typ 2-Rezeptor; B_1R: Bradykinin B_1 Rezeptor; B_2R: Bradykinin B_2 Rezeptor; CPM/N: Carboxypeptidasen M und N (führen zur Bildung der B_1R-Agonisten). (Modifiziert nach Regoli et al., 2012).

Die kardioprotektiven Effekte der ACEI und ARB werden mittlerweile auch einer erhöhten Kininwirkung zugeschrieben (Alhenc-Gelas et al., 2011). Eine direkte Aktivierung des B_2R wäre daher möglicherweise pharmakologisch interessant. Die Existenz mehrerer Aktivitätszustände des B_2R könnte Möglichkeiten für die Entwicklung neuartiger Medikamente liefern, denn ein gezieltes Eingreifen in die Signalübertragung von GPCRs ist grundsätzlich durch strukturell unterschiedliche Liganden, die nur bestimmte Signalwege aktivieren oder inhibieren, denkbar. Dieses Prinzip nennt sich „biased agonism" oder funktionelle Selektivität (Kobilka and Deupi, 2007). Um die Signaldauer zu verlängern oder zu verkürzen könnte beispielsweise nur die Rezeptorinternalisierung angesteuert werden. Eine Förderung der B_2R-Internalisierung zur längerfristigen Signalabschwächung ohne Auslösung eines G-Protein-vermittelten Signals wäre z.B. für die Behandlung des hereditären Angioödems interessant.

Erkenntnisse der letzten Jahre zeigten, dass eine Interaktion eines GPCRs mit Arrestinen nicht nur die Internalisierung sondern auch MAPK-Signalübertragung zur Folge haben kann. Es wird angenommen, dass die verschiedenen Auswirkungen durch unterschiedliche Arrestin-Konformationen zustande kommen. Jede GRK kann potentiell ein spezifisches Phosphorylierungsmuster („Barcode") am Rezeptor hinterlassen und somit eine spezifische Konformation der Arrestine induzieren („Barcode-Hypothese") (Nobles et al., 2011). Für den Angiotensin II Typ 1-Rezeptor gibt es einen sogenannten „biased agonist", das SII Angiotensin, welches im Gegensatz zu Angiotensin, keine G-Protein-abhängigen, sondern nur Arrestin-vermittelte MAPK-Kaskaden auslöst. Gleichzeitig wirkt es kompetitiv antagonistisch

auf die G-Protein-Aktivierung (Lefkowitz, 2007). Die zur Behandlung kardiovaskulärer Erkrankungen eingesetzten ARB inhibieren die durch AT II ausgelösten, vasokonstriktorischen G-Protein-abhängigen Signalwege (Alhenc-Gelas et al., 2011; Regoli et al., 2012). Würde zur Behandlung derartiger Erkrankungen allerdings ein „biased agonist" eingesetzt, könnte parallel zur Blockade des G-Protein-Signals die Arrestin-vermittelte MAPK-Signalübertragung stimuliert werden (Lefkowitz, 2007). Letztere wäre anti-apoptotisch und könnte sich im Szenario kardiovaskulärer Erkrankungen positiv auswirken (Lefkowitz, 2007). Für die Entwicklung derart spezifischer Liganden ist es von großer Bedeutung Gemeinsamkeiten und besonders Unterschiede der regulatorischen Mechanismen von GPCRs aufzuklären. Dabei wäre die Identifizierung von Aktivitätszuständen der Rezeptoren, die unterschiedlich stark mit Effektoren (G-Proteine, Arrestine) oder Regulatoren (GRKs, Arrestine) interagieren, eine große Hilfe. „Biased agonists", die diese Aktivitätszustände gezielt stabilisieren, könnten zusätzliche Möglichkeiten bieten, therapeutisch sinnvoll in die Signaltransduktion von GPCRs einzugreifen. Die Ergebnisse der vorliegenden Doktorarbeit könnten einen Beitrag zur Identifizierung solch struktureller Ansatzpunkte leisten.

5 Literaturverzeichnis

Alhenc-Gelas F, Bouby N, Richer C, Potier L, Roussel R and Marre M (2011) Kinins as therapeutic agents in cardiovascular and renal diseases. *Curr Pharm Des* **17**:2654-2662.

Ambrosio M, Fanelli F, Brocchetti S, Raimondi F, Mauri M, Rovati GE and Capra V (2010) Superactive mutants of thromboxane prostanoid receptor: functional and computational analysis of an active form alternative to constitutively active mutants. *Cellular and molecular life sciences : CMLS* **67**:2979-2989.

Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H and Sealfon SC (1998) Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J Biol Chem* **273**:10445-10453.

Birnbaumer L (2007) The discovery of signal transduction by G proteins: a personal account and an overview of the initial findings and contributions that led to our present understanding. *Biochimica et biophysica acta* **1768**:756-771.

Blaukat A, Barac A, Cross MJ, Offermanns S and Dikic I (2000) G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of Galpha(q) and Galpha(i) signals. *Mol Cell Biol* **20**:6837-6848.

Blaukat A, Pizard A, Breit A, Wernstedt C, Alhenc-Gelas F, Müller-Esterl W and Dikic I (2001) Determination of bradykinin B2 receptor in vivo phosphorylation sites and their role in receptor function. *J Biol Chem* **276**:40431-40440.

Drake MT, Shenoy SK and Lefkowitz RJ (2006) Trafficking of G protein-coupled receptors. *Circ Res* **99**:570-582.

Elliott DF, Horton EW and Lewis GP (1960) Actions of pure bradykinin. *J Physiol* **153**:473-480.

Enquist J, Skroder C, Whistler JL and Leeb-Lundberg LM (2007) Kinins promote B2 receptor endocytosis and delay constitutive B1 receptor endocytosis. *Mol Pharmacol* **71**:494-507.

Feierler J, Wirth M, Welte B, Schüssler S, Jochum M and Faussner A (2011) Helix 8 plays a crucial role in bradykinin B(2) receptor trafficking and signaling. *The Journal of biological chemistry* **286**:43282-43293.

Fredriksson R, Lagerström MC, Lundin LG and Schiöth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**:1256-1272.

Gouveia TL, Scorza FA, Silva MJ, Bandeira Tde A, Perosa SR, Arganaraz GA, Silva Mde P, Araujo TR, Frangiotti MI, Amado D, Cavalheiro EA, Silva JA, Jr. and Naffah-Mazzacoratti Mda G (2011) Lovastatin decreases the synthesis of inflammatory mediators in the hippocampus and blocks the hyperthermia of rats submitted to long-lasting status epilepticus. *Epilepsy Behav* **20**:1-5.

Greve J, Hoffmann TK, Schuler P, Lang S, Chaker A and Bas M (2011) Successful long-term treatment with the bradykinin B2 receptor antagonist icatibant in a patient with hereditary angioedema. *Int J Dermatol* **50**:1294-1295.

Jacoby E, Bouhelal R, Gerspacher M and Seuwen K (2006) The 7 TM G-protein-coupled receptor target family. *ChemMedChem* **1**:761-782.

Jansky L, Vybiral S, Pospisilova D, Roth J, Dornand J, Zeisberger E and Kaminkova J (1995) Production of systemic and hypothalamic cytokines during the early phase of endotoxin fever. *Neuroendocrinology* **62**:55-61.

Kobilka BK and Deupi X (2007) Conformational complexity of G-protein-coupled receptors. *Trends in pharmacological sciences* **28**:397-406.

Leeb-Lundberg LM, Marceau F, Müller-Esterl W, Pettibone DJ and Zuraw BL (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* **57**:27-77.

Lefkowitz RJ (2007) Seven transmembrane receptors: something old, something new. *Acta Physiol (Oxf)* **190**:9-19.

Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ and Tesmer JJ (2003) Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma. *Science* **300**:1256-1262.

Mirzadegan T, Benko G, Filipek S and Palczewski K (2003) Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry* **42**:2759-2767.

Müller MR and Rao A (2010) NFAT, immunity and cancer: a transcription factor comes of age. *Nat Rev Immunol* **10**:645-656.

Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP and Lefkowitz RJ (2011) Distinct phosphorylation sites on the beta(2)-adrenergic receptor establish a barcode that encodes differential functions of beta-arrestin. *Science signaling* **4**:ra51.

Okada T, Sugihara M, Bondar AN, Elstner M, Entel P and Buss V (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure. *J Mol Biol* **342**:571-583.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739-745.

Phagoo SB, Reddi K, Anderson KD, Leeb-Lundberg LM and Warburton D (2001) Bradykinin B1 receptor up-regulation by interleukin-1beta and B1 agonist occurs through independent and synergistic intracellular signaling mechanisms in human lung fibroblasts. *J Pharmacol Exp Ther* **298**:77-85.

Pitcher JA, Freedman NJ and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annual review of biochemistry* **67**:653-692.

Pruitt K and Der CJ (2001) Ras and Rho regulation of the cell cycle and oncogenesis. *Cancer Lett* **171**:1-10.

Regoli D, Plante GE and Gobeil F, Jr. (2012) Impact of kinins in the treatment of cardiovascular diseases. *Pharmacol Ther* **135**:94-111.

Reiter E, Ahn S, Shukla AK and Lefkowitz RJ (2012) Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol* **52**:179-197.

Reiter E and Lefkowitz RJ (2006) GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in endocrinology and metabolism: TEM* **17**:159-165.

Ritter SL and Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **10**:819-830.

Roth J and De Souza GE (2001) Fever induction pathways: evidence from responses to systemic or local cytokine formation. *Braz J Med Biol Res* **34**:301-314.

Rovati GE, Capra V and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71**:959-964.

Salon JA, Lodowski DT and Palczewski K (2011) The significance of G protein-coupled receptor crystallography for drug discovery. *Pharmacol Rev* **63**:901-937.

Shenoy SK and Lefkowitz RJ (2003) Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *The Biochemical journal* **375**:503-515.

Shenoy SK and Lefkowitz RJ (2005) Seven-transmembrane receptor signaling through beta-arrestin. *Science's STKE : signal transduction knowledge environment* **2005**:cm10.

Shukla AK, Xiao K and Lefkowitz RJ (2011) Emerging paradigms of beta-arrestin-dependent seven transmembrane receptor signaling. *Trends in biochemical sciences* **36**:457-469.

Springael JY, de Poorter C, Deupi X, Van Durme J, Pardo L and Parmentier M (2007) The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin. *Cellular signalling* **19**:1446-1456.

Teller DC, Okada T, Behnke CA, Palczewski K and Stenkamp RE (2001) Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40**:7761-7772.

Vogel R, Mahalingam M, Lüdeke S, Huber T, Siebert F and Sakmar TP (2008) Functional role of the "ionic lock"--an interhelical hydrogen-bond network in family A heptahelical receptors. *J Mol Biol* **380**:648-655.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America* **100**:10782-10787.

Wess J (1998) Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol Ther* **80**:231-264.

Willets JM, Challiss RA and Nahorski SR (2003) Non-visual GRKs: are we seeing the whole picture? *Trends Pharmacol Sci* **24**:626-633.

Yordy JS and Muise-Helmericks RC (2000) Signal transduction and the Ets family of transcription factors. *Oncogene* **19**:6503-6513.

6 Liste aller im Rahmen dieser Promotionsarbeit entstandenen Publikationen, Poster und Präsentationen

Publikationen

Leschner J[*], Wennerberg G[*], Feierler J, Bermudez M, Welte B, Kalatskaya I, Wolber G, and Faussner A (2012). Interruption of the ionic lock in the bradykinin B_2 receptor results in constitutive internalization and turns several antagonists into strong agonists. *The Journal of Pharmacology and Experimental Therapeutics.*
([*]geteilte Erstautorenschaft)

Leschner J, Ring L, Feierler J, Dinkel K, Jochum M, Faussner A (2011). Fever-like temperature modification differentially affects in vitro signaling of bradykinin B_1 and B_2 receptors. *Biological Chemistry.* 392(11):1021-9.

Vorträge

Gordon Research Seminar: Phosphorylation and G-Protein Mediated Signaling Networks, Biddeford, USA, June 2012
Leschner J.: Role of β-arrestin 1 and 2 in bradykinin B_2 receptor regulation and signaling.

29th Winter School: Proteinases and their Inhibitors, Tiers, Italy, March 2012
Leschner J., Ring L., Feierler J., Jochum M. and Faussner A.: Role of β-arrestin 1 and 2 in bradykinin B_2 receptor trafficking.

28th Winter School: Proteinases and their Inhibitors, Tiers, Italy, February 2011
Leschner J., Ring L., Feierler J., Jochum M. and Faussner A.: Fever differentially affects bradykinin B_1 and B_2 receptor signaling.

Posterpräsentationen

Gordon Research Conference: Phosphorylation and G-Protein Mediated Signaling Networks, Biddeford, USA, June 2012
Leschner J., Feierler J., Faussner A.: Role of β-arrestin 1 and 2 in bradykinin B_2 receptor regulation and signaling.

Keystone Symposia: G Protein-Coupled Receptors, Breckenridge, USA, April 2010
Leschner J., Wennerberg G., Feierler J., Schüssler S., Jochum M., Faussner A.: Comparison of the roles of highly conserved residues in the human bradykinin B_2 and B_1 receptors.

Weitere Publikationen im Rahmen der Diplomarbeit

Vilá de Muga S, Timpson P, Cubells L, Evans R, Hayes TE, Rentero C, Hegemann A, Reverter M, **Leschner J**, Pol A, Tebar F, Daly RJ, Enrich C, Grewal T (2009). Annexin A6 inhibits Ras signalling in breast cancer cells. Ongogene. 28(3):363-77.

7 Lebenslauf

8 Publikationen zur kumulativen Dissertation (A und B)

8.1 Fever-like temperature modification affects *in vitro* signaling of bradykinin B_1 and B_2 receptors (A)

8.2 Interruption of the ionic lock in the bradykinin B_2 receptor results in constitutive internalization and turns several antagonists into strong agonists (B)

A

Biol. Chem., Vol. 392, pp. 1021–1029, November 2011 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/BC.2011.095

Fever-like temperature modification differentially affects *in vitro* signaling of bradykinin B_1 and B_2 receptors

Jasmin Leschner[1], Larisa Ring[1], Jens Feierler[1], Klaus Dinkel[2], Marianne Jochum[1] and Alexander Faussner[1,*]

[1] Abteilung für Klinische Chemie und Klinische Biochemie, Ludwig-Maximilians-Universität, Nussbaumstrasse 20, D-80336 München, Germany
[2] Lead Discovery Center GmbH, Emil-Figge-Str. 76a, D-44227 Dortmund, Germany

* Corresponding author
e-mail: alexander.faussner@med.uni-muenchen.de

Abstract

The bradykinin (BK) B_2 and B_1 receptors (B_2R, B_1R) belong to the rhodopsin-like G protein-coupled receptors (GPCRs) and are involved in (patho)physiological processes such as blood pressure regulation or inflammation. They mediate the effects of the pro-inflammatory peptides bradykinin/kallidin and desArg^9-BK/desArg^{10}-kallidin, respectively. Whereas the B_2R is constitutively expressed and gets internalized upon activation, the B_1R is especially induced by inflammatory mediators and responds to stimulation with increased surface receptor numbers. Stimulation of both receptors activates phospholipase Cβ (PLCβ) and mitogen activated protein kinase (MAPK) signaling. Because inflammatory processes are characterized by heat (fever), we analyzed the effect of increased temperature (41°C vs. 37°C) on B_1R and B_2R signaling in HEK 293 and IMR 90 cells. Our results show that signaling of both receptors is temperature-sensitive, however to a different extent and with regard to the investigated pathways. Comparing PLCβ activity and Ca^{2+}-regulated signals, a temperature-dependent increase was only observed for B_1R but not for B_2R activation, whereas MAPK activities were doubled at 41°C for both receptors. Taken together, our findings suggest that the observed temperature sensitivity of B_1R-induced PLCβ activation is B_1R-specific. In contrast, the enhanced stimulation of MAPK activity under hyperthermic conditions appears to be a common phenomenon for GPCRs.

Keywords: AP-1; ERK1/2; fever; GPCR; NFAT.

Introduction

The bradykinin (BK) B_1 and B_2 receptors (B_1R and B_2R) belong to the family A (rhodopsin/β_2-adrenergic like) of G protein-coupled receptors (GPCRs) and mediate the effects of the pro-inflammatory peptides desArg^9-BK/desArg^{10}-kallidin (DABK/DAK) and BK/kallidin (Lys-BK), respectively. The latter two are often released at sites of inflammation from high- and low-molecular-weight kininogens through the action of kallikreins (Bhoola et al., 1992; Leeb-Lundberg et al., 2005) and can be converted into their desArg-derivatives by carboxypeptidases. Both receptors are involved in numerous (patho)physiological processes including blood pressure regulation, edema formation, pain sensation, inflammation, regulation of cell growth, differentiation, and mobility (Blaukat et al., 2000; Fredriksson et al., 2003; Rosenbaum et al., 2009). They have been reported to play a role in various diseases such as septic shock, atherosclerosis, and diabetes (Leeb-Lundberg et al., 2005). Although B_1R and B_2R couple to the same G proteins, $G_{q/11}$ and G_i, they differ considerably with regard to their regulation and the dynamics of their subcellular localization. B_2R is constitutively expressed in many cell types mediating the majority of physiological kinin effects under normal conditions. It gets rapidly internalized and desensitized after activation. In contrast, B_1R is expressed only weakly in healthy surroundings but is potently induced by pro-inflammatory cytokines in pathological situations such as sepsis or minor inflammation. Moreover, unlike B_2R, B_1R does not become desensitized and responds in some cell types to activation with an increase in surface receptor number rather than internalization, e.g., in IMR 90 cells (Phagoo et al., 2000; Leeb-Lundberg et al., 2005). As both kinin receptors participate in inflammatory processes by mediating the release of pro-inflammatory cytokines and recruitment of immune cells (McLean et al., 2000; Leeb-Lundberg et al., 2005; Ehrenfeld et al., 2006), the properties of the B_1R indicate sustained signaling and thus a role in the prolonged phase of the immune response with amplification of inflammatory processes (Blaukat et al., 1996; Austin et al., 1997; Marceau et al., 1998; Faussner et al., 1999; Phagoo et al., 2000; Marceau et al., 2002; Leeb-Lundberg et al., 2005). Inflammation is characterized by five cardinal symptoms: pain, redness, swelling, loss of function, and heat (Elliott et al., 1960). Infection or massive inflammation are often associated with fever and the pro-inflammatory stimuli [e.g., lipopolysaccharide, Interleukin-1β (IL-1β)] that promote B_1R expression are also effective inducers of the febrile response causing a strong increase of body temperature up to 42°C (Jansky et al., 1995; Roth and De Souza, 2001). So far, however, very little information is available on how fever affects the receptors and their signaling. Consequently, in the present study we analyzed the effect of elevated temperature (37°C vs. 41°C) on various signaling activities of the kinin receptors.

Our findings illustrate for the first time that both B_1R and B_2R are temperature-sensitive, however to a different degree with regard to distinct signal pathways. Specifically, B_1R downstream signal transduction is significantly enhanced

under hyperthermic conditions (41°C), supporting the notion that B_2R activation governs the physiological kinin effects, whereas the B_1R prolongs and amplifies these effects under pathological conditions (Dray, 1997).

Results

Heat development is one of the five cardinal signs of inflammation, which often is associated with an increase in body temperature (fever). To investigate the effects of fever on receptor signaling, we chose the kinin receptors (B_1R, B_2R), as their endogenous agonists bradykinin (BK) and desArg^{10}-kallidin (DAK) are pro-inflammatory peptides. In detail, we studied the short- and long-term effects of an increase in temperature from 37°C to 41°C on selected signaling pathways (PLCβ, NFAT, MAPK, AP-1).

Increase of B_1R- but not of B_2R-stimulated PLCβ activity at 41°C

Stimulation of B_1R as well as of B_2R results in activation of phospholipase Cβ (PLCβ), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), thereby activating protein kinase C (PKC) and increasing intracellular calcium [Ca^{2+}] concentrations. To analyze PLCβ activity, total IP accumulation was measured upon receptor activation with 1 μM of the respective agonist, in stably transfected HEK 293 cells at either 37°C or 41°C. Stimulation of B_1R with DAK at 37°C resulted in an eight-fold increase of IP accumulation and turned almost 14-fold when the stimulation was performed at 41°C (Figure 1A). In contrast, activation of B_2R with BK caused a four-fold increase over basal in PLCβ activity at 37°C, which was not further elevated by raising the temperature to 41°C (Figure 1A). Basal B_1R-mediated PLCβ activation displayed a non-significant tendency to be increased by the enhanced temperature. We also determined the EC_{50} values for total IP accumulation after B_1R stimulation at 37°C and 41°C, but in two experiments no difference was observed (0.8/0.9 nM at 37°C vs. 0.6/1.5 nM at 41°C).

Expressing the B_1R under the control of the strong cytomegalovirus (CMV) promoter and the B_2R under the control of the weaker promoter P_{min} led to comparable protein levels of the B_1R and the B_2R (1200–2000 fmol/mg protein) in our HEK 293 cells, as determined by radioligand binding assays (Faussner et al., 2009) and therefore similar signaling activity. However, to exclude that our observations resulted solely from this heterologous B_1 and B_2 receptor overexpression, we reproduced our findings in the human embryonic lung fibroblast cell line IMR 90, which endogenously expresses the B_2R (300–800 fmol/mg protein). To perform our experiments in these primary cells, the normally low B_1R levels (20–50 fmol/mg protein) were induced (100–400 fmol/mg protein) by pre-treatment with IL-1β (2.5 ng/ml) for 4 h, which should reflect a physiologic situation as IL-1β is an endogenous pyrogen involved in the febrile response (Jansky et al., 1995; Roth and De Souza, 2001). PLCβ activation via the B_1R was significantly increased in DAK-stimulated IMR 90 cells at 41°C as compared to the level at 37°C (Figure 1B), whereas B_2R-mediated PLCβ activity remained at the same level as at 37°C (data not shown). Thus, the response of both receptors in the IMR 90 cells reflects their temperature dependency found in the HEK 293 cells, demonstrating that it is not artifact caused by heterologous receptor overexpression.

We further established that high temperature treatment has no significant influence on cell surface receptor density by radioligand binding assays following incubation at 37°C and 41°C in both cell lines (data not shown).

Next we investigated whether the stronger signaling at 41°C vs. 37°C could also be observed further downstream in the IP signaling cascade.

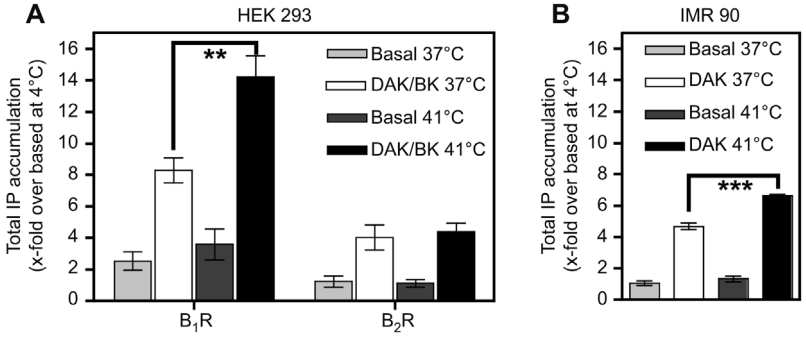


Figure 1 B_1R- and B_2R-stimulated accumulation of inositol phosphates at 37°C vs. 41°C.
(A) HEK 293 cells, stably expressing comparable amounts of either B_1R or B_2R, were stimulated in the presence of 50 mM LiCl for 30 min with 1 μM of the appropriate agonist (DAK or BK) at the indicated temperature. Thereafter, total inositol phosphate (IP) was determined as described under 'Materials and methods'. Each value represents the mean±SEM of four independent experiments performed in triplicate. The results are presented as fold increase over the IP content of identically treated control cells at 4°C. (B) IP accumulation in IMR 90 cells stimulated for 30 min with 1 μM DAK at either 37°C or 41°C. Each value represents the mean±SEM of three independent experiments performed in triplicate. The results are presented as fold increase over the IP content of identically treated control cells at 4°C. (One-way ANOVA with Bonferroni: $^{**}p<0.01$, $^{***}p<0.001$).

Elevation of B_1R- but not of B_2R-activated Ca^{2+}-dependent signaling at 41°C

When intracellular Ca^{2+} concentrations are increased, Ca^{2+} binds to calmodulin, which consequently activates the phosphatase calcineurin. Calcineurin in turn dephosphorylates the nuclear factor of activated T-cells (NFAT) transcription factor, allowing it to translocate into the nucleus and regulate gene expression by binding to its responsive elements (Muller and Rao, 2010). In our specific HEK 293 reporter cell line, the nuclear NFAT transcription factor promotes the expression of the reporter gene *Gaussia luciferase*. The NFAT reporter host cell line transiently expressing B_1R or B_2R was stimulated with DAK or BK (1 μM each), respectively, and Gaussia luciferase activity was determined 24 h later. NFAT-mediated luciferase action following B_1R stimulation with DAK at 41°C was increased two-fold as compared to the effects of a stimulation performed at 37°C. In contrast, NFAT-mediated Gaussia luciferase activity induced by B_2R stimulation with BK remained the same at 41°C as at 37°C (Figure 2). These findings are consistent with the already described observations concerning the short-term stimulation of PLCβ activity (Figure 1) and confirm that the higher temperature (41°C) increases only B_1R- but not B_2R-activated Ca^{2+}-dependent signals.

Requirement of $G_{q/11}$ and G_i for ERK1/2 activation via B_1R and B_2R

Both kinin receptors activate the MAPK signaling cascade, which is involved in major cellular processes such as cell growth, development, differentiation, and proliferation (Dhanasekaran et al., 1998; Dhillon et al., 2007; Zhang and Dong, 2007). Consequently, we analyzed the influence of increased temperature on the PKC-mediated extracellular signal-regulated kinase 1/2 (ERK1/2) pathway of the MAPK signaling cascades. It has been reported that efficient B_2R-mediated MAPK activation requires coupling to both $G_{q/11}$ and G_i (Blaukat et al., 2000). As little was known, however, about the requirements of B_1R-mediated MAPK stimulation, we first addressed the question of whether the B_1R also belongs to the group of dually coupled receptors (to $G_{q/11}$ and G_i protein subtypes) with regard to MAPK signal transmission, before analyzing the existence of a temperature dependence of B_1R- and B_2R-mediated signaling. In order to determine the contribution of $G_{q/11}$, HEK 293 cells stably expressing the B_1R or the B_2R were treated for 30 min with 5 μM BIM (bisindolylmaleimide), a potent PKC inhibitor, or for 16 h with 75 ng/ml Ptx (pertussis toxin) to inactivate G_i. Thereafter, ERK1/2 phosphorylation was determined upon stimulation of B_1R and B_2R with 1 μM of the respective receptor ligand for 5 min. Treatment with 1 μM PMA (phorbol-12-myristate-13-acetate), a strong PKC activator, for 10 min served as positive control and reference for maximal response. The DAK- and BK-induced increases in ERK1/2 phosphorylation were cut by approximately half upon pretreatment with BIM or Ptx, revealing a contribution of $G_{q/11}$ for efficient signal transduction, as well as the necessity of G_i-coupling (Figure 3A, B). The combined treatment with the PKC- and G_i-inhibiting agents had an additive effect on ERK1/2 phosphorylation for both kinin receptors, leading to an almost complete inhibition of ERK1/2 activation (Figure 3A, B). These results demonstrate that the B_1R also belongs to the group of receptors that are dually coupled to efficiently activate MAPK signaling.

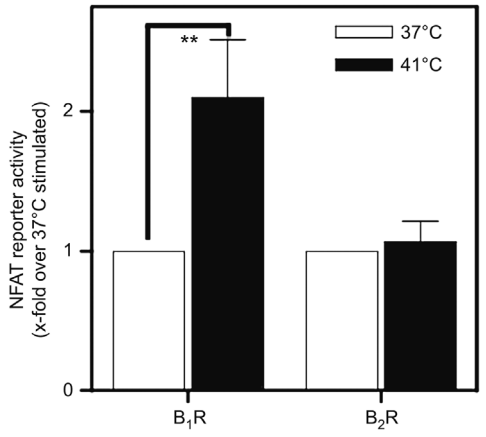


Figure 2 B_1R- and B_2R-mediated induction of NFAT-regulated Gaussia luciferase activity.

NFAT reporter gene host cells on 24-wells transiently expressing B_1R or B_2R were stimulated in DMEM containing 0.5% FCS with 1 μM of the appropriate agonist (DAK or BK) for 10 min at 37°C or 41°C. After further incubation in DMEM with 0.5% FCS for 24 h at 37°C or 41°C, respectively, luciferase activity was measured as described under 'material and methods' and normalized to stimulated luciferase activity at 37°C, which was 4±0.8-fold over basal activity following B_1R stimulation and 5±0.7-fold after B_2R activation. Values represent means±SEM of seven independent experiments performed in triplicate. (One-way ANOVA with Dunnett: $^{**}p<0.01$.)

Increase of B_1R- and B_2R-mediated ERK1/2 phosphorylation at 41°C

To determine the ERK1/2 activation potential in an inflammatory environment with increased temperatures, we firstly stimulated B_1R- or B_2R-expressing HEK 293 cells with 100 nM of the appropriate receptor agonist at 37°C and at 41°C for different time periods. Again, stimulation with PMA (1 μM) served as positive control and reference of maximal response. ERK1/2 phosphorylation was increased at 41°C as compared to the activity level at 37°C following stimulation of B_1R as well as of B_2R (Figure 4A, B). The differences in ERK1/2 activation between 37°C and 41°C were most prominent after 5 and 10 min of stimulation of both receptors. Stimulation of B_1R with DAK for 5 and 10 min resulted in a 26–31% increase of ERK1/2 phosphorylation at 41°C compared to 37°C. Triggering the B_2R also led to a significant signal elevation (18–21%) at 41°C as compared to 37°C after 5 and 10 min. A clear increase of ERK1/2 activation after stimulation of B_1R or B_2R was also found in IMR 90 cells at 41°C as compared to 37°C (Figure 4C), proving the potential physiological relevance of augmented receptor-mediated MAPK signaling activity at higher temperatures that occur during fever in inflammatory proc-

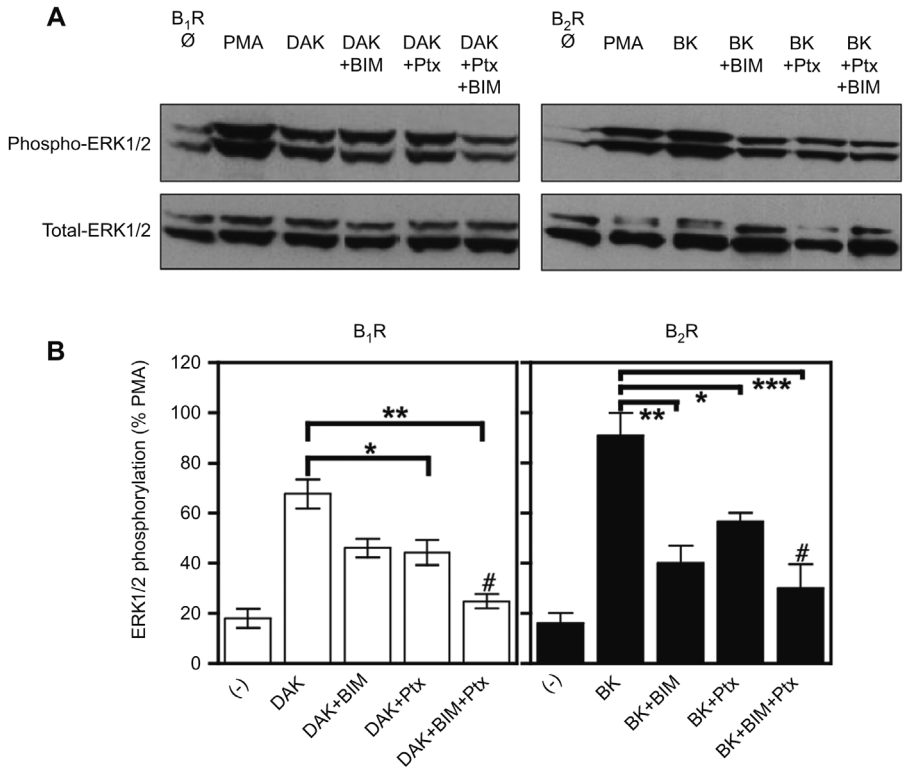


Figure 3 B_1R- and B_2R-stimulated activation of ERK1/2 involving G proteins $G_{q/11}$ and G_i.
(A) Western blot of phospho- and total-ERK1/2. HEK 293 cells stably expressing B_1R and B_2R were serum-starved overnight, pre-treated for 30 min with 5 μM BIM or for 16 h with 75 ng/ml Ptx and stimulated for 5 min with 1 μM DAK or BK as indicated. (B) Levels of phosphorylated ERK1/2 were quantified with ImageJ as described under 'Materials and methods', corrected for quantified total-ERK1/2 levels and normalized to the results obtained with 1 μM PMA (5 min) that served as reference for maximal response (=100%). Values represent means±SEM of four independent experiments. (One-way ANOVA with Bonferroni: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, # not significant compared to unstimulated.)

esses. Pre-incubation with 1 μM of Lys-des-Arg^9-[Leu^8]-BK (B_1R antagonist) or with 1 μM of Icatibant (B_2R antagonist) completely inhibited ERK1/2 phosphorylation, verifying signal transmission via the above-mentioned receptors (Figure 4A). Unlike the results observed for PLCβ-mediated and Ca^{2+}-regulated signal transduction, these findings show that with regard to ERK1/2 pathway activation, signaling activity of both kinin receptors increases with the temperature rise. This temperature sensitivity, however, is apparently a property of the ERK1/2 pathway itself, as it was basically also observed with the direct stimulation using PMA, however not reaching statistical significance (increases of 25±12% and 21±16% (n=4) of phospho-ERK1/2 levels at 41°C as compared to 37°C following PMA treatment of B_1R or B_2R-expressing cells, respectively; Figure 4A).

Elevation of AP-1-mediated reporter activity via B_1R and B_2R at 41°C

The ERK1/2/MAPK pathway was reported to possibly participate in the regulation of activator protein 1 (AP-1), a transcription factor comprised of JUN homo- or FOS-JUN heterodimers (Yordy and Muise-Helmericks, 2000; Pruitt and Der, 2001). AP-1 expression results in the regulation of key cell cycle regulators such as D-type cyclins, enabling the cell to progress through the G1 phase of the cell cycle (Yordy and Muise-Helmericks, 2000; Pruitt and Der, 2001). In order to detect temperature-sensitive long-term effects of B_1R and B_2R signaling in the context of ERK1/2 signal transduction, we investigated the temperature dependence of B_1R and B_2R concerning their ability to activate AP-1-promoted *Gaussia luciferase* gene expression.

An AP-1 reporter cell line transiently expressing the B_1R or B_2R was stimulated for 10 min at 37°C or 41°C with 1 μM of DAK or BK, respectively. After 24 h of further incubation at the same temperature, Gaussia luciferase activity was monitored. A four- to five-fold increase in enzyme activity was observed at 41°C as compared to 37°C following stimulation of both receptors (Figure 5). These findings are in agreement with our observations regarding B_1R- or B_2R-dependent ERK1/2 phosphorylation (Figure 4), rendering a participation of the ERK1/2 pathway in AP-1 transcription factor regulation feasible.

Discussion

Although pathological situations such as severe inflammation are often associated with fever, little is known on how a

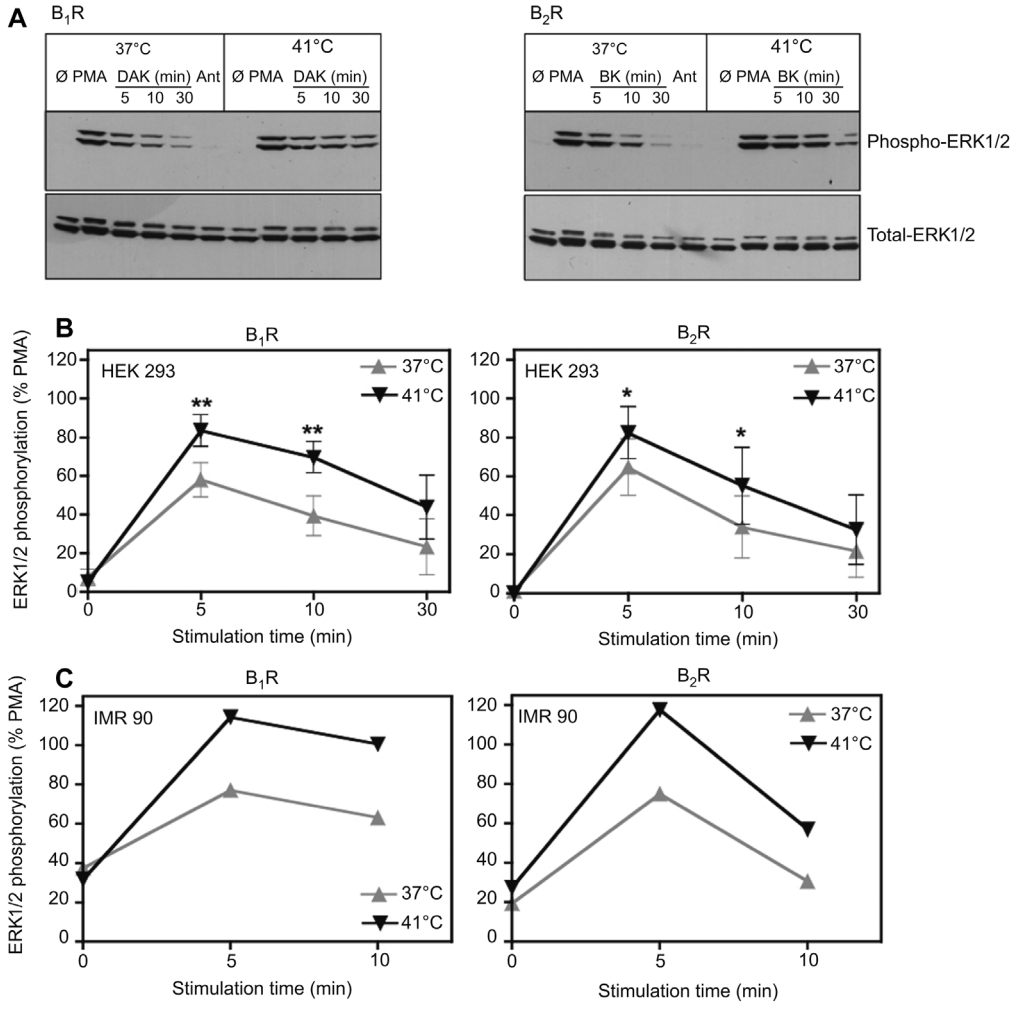


Figure 4 B_1R- and B_2R-induced ERK1/2 activation.
(A) Western blot of phospho- and total-ERK1/2. HEK 293 cells stably expressing B_1R or B_2R were serum-starved overnight, stimulated with 100 nM DAK/BK with or without pre-incubation with the appropriate antagonist (Ant) at 37°C and 41°C for the indicated times. (B) Phospho- and total-ERK1/2 protein levels were quantified and normalized as described under 'Material and methods'. Results of PMA treatment (1 μM, 5 min) served as positive control and reference for maximal response (=100%). Values represent means±SEM of four independent experiments (paired Student *t*-test: $^{**}p<0.01$, $^{*}p<0.05$). (C) ERK1/2 phosphorylation in IMR 90 cells. The experiment was performed as described for HEK 293 cells. The data of the quantification of one Western blot are shown. A second blot gave identical results.

temperature increase modifies stimulation and the following signaling of cellular membrane receptors. Such information could help to decide whether to fight against fever or to accept the temperature rise as therapeutically useful. As kinin receptors are the mediators of the effects of the pro-inflammatory kinin peptide hormones under inflammatory pathological conditions (e.g., sepsis) (Marceau et al., 2002; Leeb-Lundberg et al., 2005), these receptors appeared to be ideal to perform investigations on the temperature sensitivity of membrane receptors.

In our study, we looked at four different signaling pathways (depicted in Figure 6), comprising short-term effects of receptor activation (PI hydrolysis and ERK1/2 activation) as well as long-term effects (NFAT- and AP-1-reporter activity). Our results show that one has to differentiate between the influences of an increase in temperature on the signaling pathways themselves and effects that are specific for a receptor activating these pathways.

ERK1/2 phosphorylation and AP-1-regulated reporter gene activation were significantly enhanced at 41°C as compared to 37°C upon stimulation of both kinin receptors. This temperature dependency was basically also observed when the ERK1/2 pathway was stimulated with the phorbolester PMA, however not reaching statistical significance [with increases of 21–24% in ERK1/2 phosphorylation at 41°C as compared to 37°C (see Figure 4) and 1.7±0.3-fold enhanced AP-1 reporter activity at 41°C over 37°C (see Figure 5)], i.e., without the initial receptor and G protein activation step. The augmented ERK1/2 activation at 41°C may, therefore, not be specific for the kinin receptors, but can also most likely be

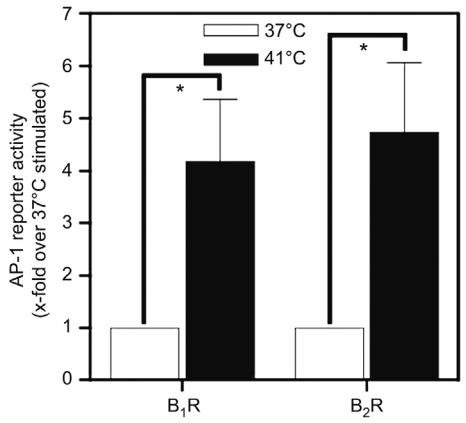


Figure 5 B_1R- and B_2R-mediated induction of AP-1-regulated Gaussia luciferase activity.
AP-1 reporter gene host cells on 24-wells transiently expressing B_1R or B_2R were stimulated in DMEM containing 0.5% FCS with 1 μM of the appropriate agonist (DAK or BK) for 10 min at 37°C or 41°C. After further incubation in DMEM with 0.5% FCS for 24 h at 37°C or 41°C, respectively, luciferase activity was measured as described under 'Material and methods' and normalized to stimulated luciferase activity at 37°C, which was 6±2-fold over basal activity following B_1R stimulation and 3±1-fold after B_2R activation. Values represent means±SEM of five independent experiments performed in triplicate. (One-way ANOVA with Dunnett: $^*p<0.05$.)

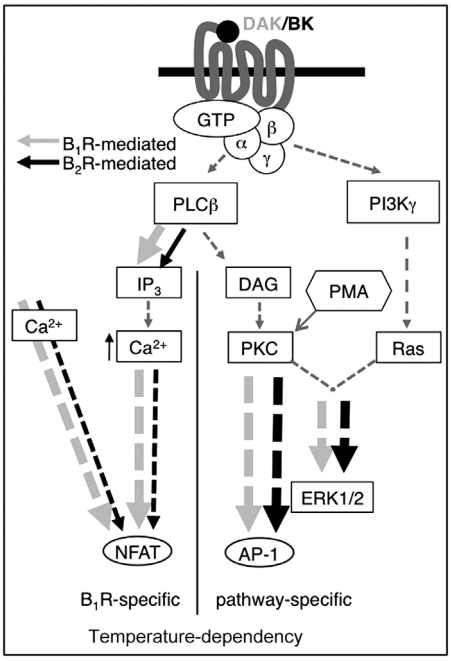


Figure 6 Overview of B_1R- and B_2R-mediated pathways at 37°C vs. 41°C.
Bold arrows indicate increased signal activity at 41°C as compared to 37°C. Light gray arrows show B_1R-specific temperature dependence, while black arrows show pathway-specific temperature dependency. Both kinin receptors are temperature-sensitive. However, mainly B_1R signaling activity is strongly enhanced at elevated temperatures.

detected for other GPCRs activating this pathway. The effect of a temperature rise on ERK1/2 activation was also apparently not dependent on the cell type as it was assessed for the kinin receptors heterologously expressed in HEK 293 cells and for those expressed endogenously in IMR 90 fibroblasts as well (see Figure 4C).

In contrast, a temperature sensitivity of PI hydrolysis or the NFAT-regulated reporter activity was found only after stimulation of the B_1R, but not of the B_2R (see Figure 1 and Figure 2). This indicates that the responses of these two pathways are not generally enhanced with temperature, but solely when activated via specific receptors such as the B_1R. As shown in different cell types (HEK 293, IMR 90 fibroblasts), the temperature sensitivity is not a common property of the pathway, but rather an intrinsic quality of the respective receptor, in our case of the B_1R.

Although both kinin receptors apparently couple to the same signaling pathways, thereby activating identical second messengers and kinase cascades, they otherwise exhibit quite contrasting regulatory patterns. The B_2R gets rapidly phosphorylated, desensitized, and internalized upon stimulation with an agonist. In contrast, the B_1R does not become phosphorylated and does not undergo agonist-induced desensitization (Leeb-Lundberg et al., 2005). Consequently, while the B_2R induces a transient increase in PI hydrolysis and is only weakly dependent on extracellular Ca^{2+}, the B_1R promotes sustained signaling and is significantly dependent on extracellular Ca^{2+} (Tropea et al., 1993; Leeb-Lundberg et al., 2005). Moreover, in contrast to the B_2R, which is constitutively expressed in many cells, the B_1R is induced preferentially under pathological conditions by inflammatory cytokines (Leeb-Lundberg et al., 2005).

All these differences led to the assumption that the B_2R acts during the acute phase of an inflammation or other pathological situations, whereas the B_1R takes over as (patho)physiologically dominating kinin receptor in the chronic phase after being expressed in sufficient amounts (Enquist et al., 2007). The observed different temperature sensitivity of both receptors in our experiments further strengthens this notion of a switch from B_2R to enhanced B_1R signal transduction in an inflammatory situation with elevated temperatures. Future studies will have to determine how the specifically increased B_1R signaling is achieved, whether through enhanced efficacy as a guanine-nucleotide exchange factor for G proteins or via other temperature-sensitive factors (e.g., Ca^{2+} channels translocated to the plasma membrane), which may exert their augmenting effect sufficiently only with receptors that do not become immediately desensitized, such as the B_1R.

Furthermore, several studies have demonstrated that kinin receptors are involved in leukocyte migration, and are upregulated on neutrophils in a variety of inflammatory disorders, e.g., asthma and rheumatoid arthritis (Bertram et al., 2007). The temperature sensitivity of the kinin receptors, as observed by us, is therefore most likely of therapeutic relevance. In this context, it is of interest that Gouveia et al.

recently reported that the application of the drug lovastatin to rats with pilocarpine-induced status epilepticus resulted in a reduction of body temperature, in decreased IL-1β and IL-6 levels, and importantly in a decline of B_1R expression. The authors concluded that normalization of body temperature could be an approach of neuroprotection in generalized convulsive forms of status epilepticus (Gouveia et al., 2011). Assuming that B_1R signaling is increased during status epilepticus due to higher temperatures, neuroprotection might occur partly because of a diminution of B_1R activity at normal physiological temperatures.

Interestingly, it is worth mentioning that GPCRs cannot only be affected in their signaling properties by the surrounding temperature as shown herein, but may even act as a temperature sensor as has recently been reported for the GPCR rhodopsin in *Drosophila* larvae (Shen et al., 2011).

Taken together, our findings illustrate, to the best of our knowledge for the first time, that some GPCR signaling pathways (e.g., ERK1/2, AP-1) are significantly affected by the (patho)physiologically relevant temperature increase from 37°C to 41°C and thus most likely will respond to all activating GPCRs with a stronger signal at the higher temperature. In contrast, other pathways (e.g., PI hydrolysis, NFAT) are basically insensitive to (patho)physiological temperature alterations. An observed effect of temperature on a response in these cases is therefore specific for the activating receptor, as demonstrated for the B_1R in our experiments. Since the signaling activity of the B_1R is specifically increased in hyperthermic surroundings in addition to its upregulation under fever-associated inflammatory conditions, targeting this GPCR might be of special therapeutic relevance for the decision of whether or not to counteract fever.

Materials and methods

Materials

Flp-In™ TREx-293 (HEK 293) cells were bought from Invitrogen (Karlsruhe, Germany) and IMR 90 cells from ATCC (Manassas, USA). [2,3-prolyl-3,4-^{3}H]bradykinin (80 Ci/mmol) and myo-[2-^{3}H]inositol (22 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, USA), Kallidin[des-Arg10],[3,4-Prolyl-3,4-^{3}H] (70 Ci/mmol) from Hartmann Analytic (Braunschweig, Germany). Bradykinin and desArg^{10}-Kallidin were synthesized by Bachem (Heidelberg, Germany). Fugene was from Roche (Mannheim, Germany), EcoTransfect from Oz Biosciences (Marseille, France). Poly-D-Lysine, captopril, 1.10-phenanthroline, bacitracin and pertussis toxin were obtained from Sigma-Aldrich (Taufkirchen, Germany). Cell culture media and additions were from PAA Laboratories (Coelbe, Germany), Opti-MEM I from Gibco (Darmstadt, Germany). Bisindolylmaleimide I, phorbol-12-myristate-13-acetate and ionomycin were purchased from Calbiochem (Darmstadt, Germany). Monoclonal phospho-ERK1/2- and ERK1/2-antibodies, as well as the peroxidase-labeled horse anti-mouse IgG-antibody were supplied by Cell Signaling Technology (Massachusetts, USA).

Gene expression and cell culture

The sequence of the B_1R, codon-optimized for expression in human cells and synthesized by GeneArt (Regensburg, Germany) and the sequence of the B_2R starting with the third encoded Met (Hess et al., 1992), were cloned into the HindIII and XhoI sites of the pcDNA5/FRT/TO vector (Invitrogen). A hemagglutinin-tag (MGYPYDVPDYAGS) preceded the receptor sequences. For stable expression, the Flp-In system with the host cell line Flp-In™ TREx-293 (HEK 293) and recombinase-directed insertion of the plasmid harboring the gene of interest was applied. The HEK 293 cells cultivated in Dulbecco's modified Eagle's Medium (DMEM) with 10% FCS and penicillin (100 U/ml)/streptomycin (100 μg/ml) were transfected using the transfection reagent Fugene according to the manufacturer's instructions. Selection of stable clones was achieved using hygromycin B (250 μg/ml). IMR 90 cells were cultured in minimum essential medium Eagle (MEM) with Earle's Salts (10% FCS, 1% glutamine). To generate reporter gene host cell lines, HEK 293 cells were stably transfected with a pcDNA3.1/G418 vector (Invitrogen), which had the CMV promoter replaced by the respective transcriptional response elements inserted in multiple copies (4×GGAGGAAAAACTGTTTCATACAGAAAGGCGT for NFAT, 6×AGCCTGACGTCAGAG for AP-1) followed by the *Gaussia luciferase* gene cloned into the EcoRI and XhoI sites of the vector (Larissa Ring et al., unpublished results). Cells were selected in the presence of G418 (1 mg/ml).

Radioligand binding assays

Monolayers of IMR 90 cells or stably transfected HEK 293 cells in 24-wells were rinsed twice with ice-cold PBS and incubated on ice with the respective radiolabeled agonist 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, degradation inhibitors for B_1R: 0.5 mM bacitracin, 0.02 mM 1.10-phenanthroline, 100 μM captopril; degradation inhibitors for B_2R: 2 mM bacitracin, 0.8 mM 1.10-phenanthroline, 100 μM captopril) for 90 min. After washing with ice-cold PBS, surface-bound [^{3}H]-labeled ligand was dissociated by incubation (10 min, 4°C) with 200 μl of ice-cold 0.5 M NaCl/0.2 M acetic acid solution, pH 2.7, and counted in a β-counter after transfer to a scintillation vial and addition of scintillation liquid. Nonspecific binding was determined in the presence of a 1000-fold excess of non-radiolabeled ligand.

Determination of total inositol phosphate (IP) release

IMR 90 cells or stably transfected HEK 293 cells, plated on 12- or 24-wells, respectively, were labeled overnight with 1 μCi [^{3}H]inositol/ml in 500/250 μl of Opti-MEM I. Cells were rinsed twice with ice-cold PBS and pre-incubated for 90 min on ice in incubation buffer supplemented with 50 mM LiCl with or without 1 μM of the indicated ligand. To determine EC_{50} values, cells were pre-incubated with increasing concentrations (10^{-12}–10^{-6} M) of ligand solution at 4°C. Cells were then stimulated for 30 min at either 37°C or 41°C. Stimulation was terminated by exchange of ligand solution for 1.5/0.75 ml of ice-cold 20 mM formic acid solution. After 60 min, the amount of total IP in the formic acid extract was determined by column chromatography using AG 1-X8 columns (Bio-Rad, Munich, Germany) as described earlier (Faussner et al., 2009).

Determination of ERK1/2 phosphorylation

Confluent IMR 90 cells or HEK 293 cells on 6-well plates expressing the respective kinin receptor were serum-starved by incubation in Opti-MEM I. After 24 h they were stimulated with 1 μM of PMA, the given concentrations of DAK or BK for the indicated times at the indicated temperature with or without pre-incubation with 1 μM Lys-des-Arg^9-[Leu^8]-BK, 1 μM Icatibant, 5 μM BIM

for 30 min, or pertussis toxin (75 ng/ml) for 16 h. Subsequently, the monolayers were washed with ice-cold PBS, scraped in lysis buffer (10 mM Tris-HCl, pH 7,4, 150 mM NaCl, 25 mM KCl, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (complete Mini, EDTA-free; Roche, Mannheim, Germany) and centrifuged at 14 000 rpm for 15 min at 4°C. Cell lysates were mixed with NuPAGE LDS Sample Buffer (Invitrogen) supplemented with DTT (0.1 M) and heated at 99°C for 10 min. Equal amounts of protein were separated by 4–12% SDS-PAGE and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). After 60 min in blocking buffer [5% milk powder in Tris-buffered saline with 0.1% Tween 20 (TBST)] the primary antibodies (phospho-ERK1/2, ERK1/2, monoclonal, Cell Signaling) were added for 60 min in blocking buffer. After washing with TBST the secondary peroxidase-labeled horse anti-mouse IgG (Cell Signaling) was added for 60 min in blocking buffer, followed by thorough washing and visualization of the immuno-labeled proteins using Western blot Chemiluminescence Reagent Plus (Roche) on X-ray hyperchemiluminescence films (Hyperfilm ECL, GE Healthcare). Band densities were quantified using the according software, ImageJ (Abramoff et al., 2004; Rasband, 1997–2011). Protein levels were normalized to the corresponding PMA-stimulated phospho-ERK1/2 levels at 37°C as maximum responses.

Reporter gene assays

Reporter host cell lines on 24-wells were transiently transfected with B_1R or B_2R using the transfection reagent EcoTransfect (0.2 μg DNA/0.6 μl EcoTransfect per well) and incubated overnight in complete cell culture medium. The next day, cells were induced with tetracyclin (0.5 μg/ml) in medium with reduced FCS (0.5%). 24 h later, cells were stimulated for 10 min at 37°C or 41°C with 1 μM of the respective ligand (DAK/BK) and further incubated at 37°C or 41°C overnight. 1 μM PMA±1 μM ionomycin served as positive controls as indicated. 24 h post stimulation, 75 μl of the cell culture supernatant, containing secreted Gaussia luciferase, was transferred to a 96-well plate. Upon addition of 25 μl of Gaussia luciferase substrate (5.72 μM Coelenterazin, 2.2 mM Na_2EDTA, 0.22 M K_xPO_4, pH 5.1; 0.44 mg/ml BSA, 1.1 M NaCl, 1.3 mM NaN_2, pH 5.0), its activity was monitored within 1 min in a Transluminator DarkReader (Tecan safire2, Clare Chemical Research, Dolores, USA), followed by quantification using XFluor4 Safire2 according software (Tecan, Crailsheim, Germany).

Protein quantification

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

Data analysis

All data analysis was performed with GraphPad Prism for Macintosh, Version 4.0c (GraphPad Software, Inc., San Diego, CA, USA). Data were assessed by appropriate analysis of variance (ANOVA or Student's *t*-test), with *post hoc* analysis as indicated (Bonferroni or Dunnett).

Acknowledgments

We are grateful to C. Seidl and H. Grondinger for excellent technical assistance.

References

Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image processing with image. J. Biophotonics Int. *11*, 36–42.

Austin, C.E., Faussner, A., Robinson, H.E., Chakravarty, S., Kyle, D.J., Bathon, J.M., and Proud, D. (1997). Stable expression of the human kinin B1 receptor in Chinese hamster ovary cells. Characterization of ligand binding and effector pathways. J. Biol. Chem. *272,* 11420–11425.

Bertram, C., Misso, N.L., Fogel-Petrovic, M., Figueroab, C., Thompsona, P.J., and Bhoolaa, K.D. (2007). Comparison of kinin B_1 and B_2 receptor expression in neutrophils of asthmatic and non-asthmatic subjects. Int. Immunopharmacol. *7,* 1862–1868.

Bhoola, K.D., Figueroa, C.D., and Worthy, K. (1992). Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol. Rev. *44,* 1–80.

Blaukat, A., Alla, S.A., Lohse, M.J., and Muller-Esterl, W. (1996). Ligand-induced phosphorylation/dephosphorylation of the endogenous bradykinin B_2 receptor from human fibroblasts. J. Biol. Chem. *271,* 32366–32374.

Blaukat, A., Barac, A., Cross, M.J., Offermanns, S., and Dikic, I. (2000). G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of Gα(q) and Gα(i) signals. Mol. Cell Biol. *20,* 6837–6848.

Dhanasekaran, N., Tsim, S.T., Dermott, J.M., and Onesime, D. (1998). Regulation of cell proliferation by G proteins. Oncogene *17,* 1383–1394.

Dhillon, A.S., Hagan, S., Rath, O., and Kolch, W. (2007). MAP kinase signalling pathways in cancer. Oncogene *26,* 3279–3290.

Dray, A. (1997). Kinins and their receptors in hyperalgesia. Can. J. Physiol. Pharmacol. *75,* 704–712.

Ehrenfeld, P., Millan, C., Matus, C.E.,Figueroa, J.E., Burgos, R.A., Nualart, F., Bhoola, K.D., and Figueroa, C.D. (2006). Activation of kinin B_1 receptors induces chemotaxis of human neutrophils. J. Leukoc. Biol. *80,* 117–124.

Elliott, D.F., Horton, E.W., and Lewis, G.P. (1960). Actions of pure bradykinin. J. Physiol. *153,* 473–480.

Enquist, J., Skroder, C., Whistler, J.L., and Leeb-Lundberg, L.M. (2007). Kinins promote B_2 receptor endocytosis and delay constitutive B_1 receptor endocytosis. Mol. Pharmacol. *71,* 494–507.

Faussner, A., Bathon, J.M., and Proud, D. (1999). Comparison of the responses of B_1 and B_2 kinin receptors to agonist stimulation. Immunopharmacology *45,* 13–20.

Faussner, A., Wennerberg, G., Schussler, S., Feierler, J., Seidl, C., Jochum, M., and Proud, D. (2009). Alanine screening of the intracellular loops of the human bradykinin B receptor – effects on receptor maintenance, G protein activation and internalization. FEBS J. *276,* 3491–3503.

Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., and Schioth, H.B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol. Pharmacol. *63,* 1256–1272.

Gouveia, T.L., Scorza, F.A., Silva, M.J., Bandeira Tde, A., Perosa, S.R., Argañaraz, G.A., Silva Mde, P., Araujo, T.R., Frangiotti, M.I., Amado, D., et al. (2011). Lovastatin decreases the synthesis of inflammatory mediators in the hippocampus and blocks the hyperthermia of rats submitted to long-lasting status epilepticus. Epilepsy Behav. *20,* 1–5.

Hess, J.F., Borkowski, J.A., Young, G.S., Strader, C.D., and Ransom, R.W. (1992). Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. Biochem. Biophys. Res. Commun. *184,* 260–268.

Jansky, L., Vybiral, S., Pospisilova, D., Roth, J., Dornand, J., Zeisberger, E., and Kamínková, J. (1995). Production of systemic and hypothalamic cytokines during the early phase of endotoxin fever. Neuroendocrinology *62,* 55–61.

Leeb-Lundberg, L.M., Marceau, F., Muller-Esterl, W., Pettibone, D.J., and Zuraw, B.L. (2005). International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol. Rev. *57,* 27–77.

Marceau, F., Hess, J.F., and Bachvarov, D.R. (1998). The B_1 receptors for kinins. Pharmacol. Rev. *50,* 357–386.

Marceau, F., Sabourin, T., Houle, S., Fortin, J.P., Petitclerc, E., Molinaro, G., and Adam A. (2002). Kinin receptors: functional aspects. Int. Immunopharmacol. *2,* 1729–1739.

McLean, P.G., Ahluwalia, A., and Perretti, M. (2000). Association between kinin B_1 receptor expression and leukocyte trafficking across mouse mesenteric postcapillary venules. J. Exp. Med. *192,* 367–380.

Muller, M.R. and Rao, A. (2010). NFAT, immunity and cancer: a transcription factor comes of age. Nat. Rev. Immunol. *10,* 645–656.

Phagoo, S.B., Yaqoob, M., Herrera-Martinez, E., McIntyre, P., Jones, C., Burgess, G.M. (2000). Regulation of bradykinin receptor gene expression in human lung fibroblasts. Eur. J. Pharmacol. *397,* 237–246.

Pruitt, K. and Der, C.J. (2001). Ras and Rho regulation of the cell cycle and oncogenesis. Cancer Lett. *171,* 1–10.

Rasband, W.S. (1997–2011). ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imageJ.nih.gov/ij/.

Rosenbaum, D.M., Rasmussen, S.G., and Kobilka, B.K. (2009). The structure and function of G-protein-coupled receptors. Nature *459,* 356–363.

Roth, J. and De Souza, G.E. (2001). Fever induction pathways: evidence from responses to systemic or local cytokine formation. Braz. J. Med. Biol. Res. *34,* 301–314.

Shen, W.L., Kwon, Y., Adegbola, A.A., Luo, J., Chess, A., and Montell, C. (2011). Function of rhodopsin in temperature discrimination in Drosophila. Science *331,* 1333–1336.

Tropea, M.M., Gummelt, D., Herzig, M.S., and Leeb-Lundberg, L.M. (1993). B_1 and B_2 kinin receptors on cultured rabbit superior mesenteric artery smooth muscle cells: receptor-specific stimulation of inositol phosphate formation and arachidonic acid release by des-Arg9-bradykinin and bradykinin. J. Pharmacol. Exp. Ther. *264,* 930–937.

Yordy, J.S. and Muise-Helmericks, R.C. (2000). Signal transduction and the Ets family of transcription factors. Oncogene *19,* 6503–6513.

Zhang, Y. and Dong, C. (2007). Regulatory mechanisms of mitogen-activated kinase signaling. Cell. Mol. Life Sci. *64,* 2771–2789.

Received May 20, 2011; accepted July 13, 2011; previously published online August 29, 2011

B

1521-0103/344/1/85–95$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.112.199190
J Pharmacol Exp Ther 344:85–95, January 2013

Interruption of the Ionic Lock in the Bradykinin B_2 Receptor Results in Constitutive Internalization and Turns Several Antagonists into Strong Agonists[S]

Jasmin Leschner, Goeran Wennerberg, Jens Feierler, Marcel Bermudez, Benjamin Welte, Irina Kalatskaya, Gerhard Wolber, and Alexander Faussner

Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Ludwig-Maximilians-Universitaet, Muenchen (J.L., G.W., J.F., B.W., A.F.), Institut für Pharmazie, Pharmazeutische Chemie, Freie Universitaet Berlin, Berlin, Germany (M.B., G.W.); and Ontario Institute for Cancer Research, MaRS Centre, Toronto, Ontario, Canada (I.K.)

Received August 9, 2012; accepted October 16, 2012

ABSTRACT

The DRY motif with the highly conserved R3.50 is a hallmark of family A G protein-coupled receptors (GPCRs). The crystal structure of rhodopsin revealed a salt bridge between $R135^{3.50}$ and another conserved residue, $E247^{6.30}$, in helix 6. This ionic lock was shown to maintain rhodopsin in its inactive state. Thus far, little information is available on how interruption of this ionic bond affects signaling properties of nonrhodopsin GPCRs, because the focus has been on mutations of R3.50, although this residue is indispensable for G protein activation. To investigate the importance of an ionic lock for overall receptor activity in a nonrhodopsin GPCR, we mutated $R128^{3.50}$ and $E238^{6.30}$ in the bradykinin (BK) B_2 receptor (B_2R) and stably expressed the constructs in HEK293 cells. As expected, mutation of R3.50 resulted in lack of G protein activation. In addition, this mutation led to considerable constitutive receptor internalization. Mutation of E6.30 (mutants E6.30A and E6.30R) also caused strong constitutive internalization. Most intriguingly, however, although the two E6.30 mutants displayed no increased basal phosphatidylinositol hydrolysis, they gave a response to three different B_2R antagonists that was almost comparable to that obtained with BK. In contrast, swapping of R3.50 and E6.30, thus allowing the formation of an inverse ionic bond, resulted in rescue of the wild type phenotype. These findings demonstrate for the first time, to our knowledge, that interruption of the ionic lock in a family A GPCR can have distinctly different effects on receptor internalization and G protein stimulation, shedding new light on its role in the activation process.

Introduction

G protein-coupled receptors (GPCRs) represent the largest superfamily of transmembrane receptors in the human genome; 25–50% of all marketed drugs act directly or indirectly via members of this family. Nonetheless, their potential for future drug development is not fully used as a detailed elucidation of GPCR regulatory mechanisms; in particular, their receptor specificity is still lacking (Vassilatis et al., 2003; Jacoby et al., 2006; Overington et al., 2006). Rhodopsin-like family A GPCRs are characterized by a few highly conserved amino acid motifs (Fig. 1) that are assumed to play a crucial role for structure and/or function (Nygaard et al., 2009; Salon et al., 2011). The dark-state crystal structure of bovine rhodopsin (PDB: 1U19) displayed a salt bridge between arginine 3.50 [R3.50, denoted according to the Ballesteros/Weinstein numbering (Ballesteros et al., 1998)] of the E/DRY motif at the cytosolic end of transmembrane domain 3 (TM3) and glutamate 6.30 (E6.30) in helix 6 (Palczewski et al., 2000). This interaction is also known as ionic lock and has been reported to maintain the inactive rhodopsin conformation by connecting TM3 and TM6 (Teller et al., 2001; Okada et al., 2004; Vogel et al., 2008). R3.50 is the most conserved residue in family A GPCRs (96%), in which an acidic residue in position 6.30 is also quite common (32%) (Mirzadegan et al., 2003; Springael et al., 2007). However, with regard to the latter, there are large differences among subfamilies: it is found in almost all amine GPCRs (>90% E/D6.30) but occurs rarely in peptide GPCRs (<7%). Of surprise, little information is available on the importance of the ionic lock for the overall activity status in these nonrhodopsin GPCRs. This is mainly attributable to the fact that most investigations focused on R3.50 in the DRY motif instead of on the acidic residue in position 6.30 (Scheer et al., 1996; Ballesteros et al., 1998; Rovati et al., 2007). R3.50, however, is per se indispensable for G protein activation (Rovati et al., 2007; Schneider

This work was supported by the Förderprogramm für Forschung und Lehre of the Medical Faculty of the Ludwig-Maximilians-University of Munich, Munich, Germany (to B.W.).
J.L. and G.W. (Goeran Wennerberg) contributed equally to this work.
dx.doi.org/10.1124/jpet.112.199190.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: B9430, D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-Arg-OH; BK, bradykinin; B_2Rwt, bradykinin B_2 receptor wild type; E6.30, glutamate 6.30; GPCR, G protein-coupled receptor; GRK, GPCR kinase; JSM10292, (1-{4-Methyl-3-[2-methyl-4-(4-methyl-2H-pyrazol-3-yl)-quinolin-8-yloxymethyl]-pyridin-2-ylmethyl}-3-trifluoromethyl-1H-pyridin-2-one); MAPK, mitogen-activated protein kinase; NPC17731, (D)Arg-[Hyp^3,(D)HypE(*trans*-propyl)^7-Olc^8]-bradykinin; PAO, phenylarsine oxide; TM3, transmembrane domain 3.

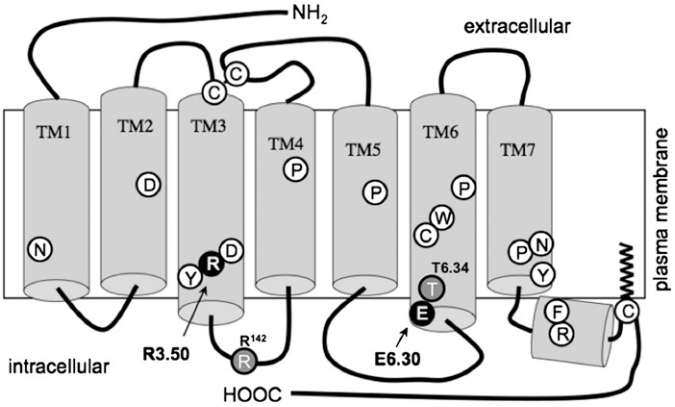


Fig. 1. Schematic two-dimensional representation of a family A GPCR. Highly conserved residues are shown as white circles and the one letter code for amino acids in black. The two residues involved in the formation of the ionic lock and residues T6.34 and Arg142 (see *Discussion*) are depicted in black or gray circles, respectively, with white letters. The Ballesteros/Weinstein numbering (Ballesteros et al., 1998) has been used for identification of residues.

et al., 2010). Therefore, unfortunately, mutation of R3.50 and G protein-mediated processes are not suitable to determine the consequences of a disruption of the ionic lock on the receptor activity status. In contrast, receptor internalization is also an important process of most activated nonrhodopsin GPCRs and has been reported to be G protein independent (Shenoy and Lefkowitz, 2005; Shukla et al., 2011). Thus, it is astonishing that the impact of a mutation of R3.50 and the disruption of the ionic lock on this important regulatory mechanism has not been investigated yet.

The human bradykinin B_2 receptor (B_2R) is a member of the family A GPCRs. Moreover, it is one of the very few peptide GPCRs with an acidic glutamate in position 6.30 and, thus, is suited for studying the ionic lock in a nonrhodopsin GPCR. The B_2R is ubiquitously expressed in almost all cells and tissues and plays an important role in a variety of physiologic processes comprising vasodilatation, edema formation, natriuresis, and hyperalgesia (Leeb-Lundberg et al., 2005). Its stimulation results in activation of the G proteins $G_{q/11}$ and G_i and of G protein–dependent mitogen-activated protein kinase (MAPK) cascades (Blaukat et al., 2000; Leschner et al., 2011). Ligand-mediated receptor internalization is important for the regulation of B_2R signaling. It is initiated by phosphorylation of serine/threonine residues in the B_2R C terminus, which leads to the recruitment of β-arrestins and ends with the sequestration of the receptor into intracellular compartments (Leeb-Lundberg et al., 2005).

In recent years, it has become clear that multiple conformations of a single GPCR exist that are coupled in different ways to the various effects controlled by the respective receptor (Rosenbaum et al., 2009; Salon et al., 2011) and may depend on the type of ligand bound. Therefore, we hypothesized that the ionic lock and the surrounding amino acid network might play distinct roles in G protein activation and receptor internalization. We used the B_2R to test our hypothesis and were able to show for the first time that interruption of the ionic lock differentially affects receptor internalization and G protein–mediated signaling. Moreover, disrupting the ionic lock by mutating only a single amino acid (E6.30) completely altered the impact of several antagonists on these processes. Our findings argue for a multistep process of GPCR activation, in which each step might have a different sensitivity for agonists and antagonists. This new insight into GPCR activation might contribute to a more efficient drug design.

Materials and Methods

Materials. Flp-In TREx-293 (HEK293) cells and Opti-MEM I serum-free medium were obtained from Invitrogen (Karlsruhe, Germany); [2,3-prolyl-3,4-^{3}H]bradykinin (80 Ci/mmol), *myo*-[2-^{3}H] inositol (22 Ci/mmol), and [prolyl-3,4-^{3}H]NPC17731 [(D)Arg-[Hyp^3,(D)HypE(*trans*-propyl)^7-Olc^8]-bradykinin; 48.5 Ci/mmol] were from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{32}P]Phosphoric acid was delivered by Hartmann Analytic (Braunschweig, Germany). BK was purchased from Bachem (Heidelberg, Germany). B9430 [D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-Arg-OH], icatibant, and JSM10292 (1-{4-Methyl-3-[2-methyl-4-(4-methyl-2H-pyrazol-3-yl)-quinolin-8-yloxymethyl]-pyridin-2-ylmethyl}-3-trifluoromethyl-1H-pyridin-2-one) were generous gifts from Dr. L. Gera and J. Stewart (Denver, CO) and Jerini (Berlin, Germany), respectively. Roche (Mannheim, Germany) delivered FuGeneHD, complete mini EDTA-free protease inhibitor tablets and the rat monoclonal anti-hemagglutinin (HA)–peroxidase high-affinity antibody (3F10). EZview red anti-HA affinity gel, poly-D-lysine, captopril, 1,10-phenanthroline, and bacitracin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum, DMEM, penicillin/streptomycin, and hygromycin B were obtained from PAA Laboratories (Coelbe, Germany). Primers were synthesized by MWG-Biotech (Ebersberg, Germany) and delivered desalted and lyophilized.

Gene Mutagenesis, Expression, and Cell Culture. Standard polymerase chain reactor techniques with accordingly designed primers and the bradykinin B_2 receptor (B_2R) gene as template were used to generate point-mutants of the B_2R. The coding sequences of the B_2Rwt and the mutants started with the third encoded Met (Hess et al., 1992) and were cloned into the HindIII and XhoI sites of the pcDNA5/FRT/TO vector (Invitrogen). At the N terminus, a single hemagglutinin tag (MGYPYDVPDYAGS), with the last two amino acids (Gly-Ser) of the tag deriving from the insertion of a BamHI restriction site, preceded the receptor sequences. For stable, inducible expression of the constructs, we used the Flp-In TREx-293 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 (HEK293) through the transient expression of recombinase pOG44. HEK293 cells, cultivated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin, were transfected using the transfection reagent FuGeneHD according to the instructions of the manufacturer. Single stably expressing clones resulted from selection with 250 μg/ml hygromycin B. Receptor expression in these cells was induced by addition of 0.5 μg/ml tetracycline 1–2 days before the experiment. For experiments requiring repeated rinsing of the cells, poly-D-lysine-treated (0.01% in phosphate-buffered saline [PBS]) cell culture dishes were used to ensure adherence.

Equilibrium Binding Experiments at 4°C and 37°C. The dissociation constant (K_d) was determined with [^{3}H]BK as described previously (Faussner et al., 2004). For determination of the equilibrium binding affinity constant at 37°C, receptor sequestration was inhibited by pretreatment of the cells with 100 μM phenylarsine oxide (PAO) for 5 minutes at 37°C (Faussner et al., 2004). Cell monolayers in 48 wells were incubated either on ice for 90 minutes or at 37°C for 30 minutes 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) with degradation inhibitors (2 mM bacitracin, 0.8 mM 1,10-phenanthroline, and 100 μM captopril) containing increasing concentrations of up to 30 nM [^{3}H]BK. Thereafter, cells were rinsed, bound [^{3}H]BK dissociated by a 10-minutes 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 addition of scintillation fluid. Nonspecific binding was determined with a 1000-fold excess of unlabeled BK and subtracted from total binding to give receptor-specific binding.

[^{3}H]BK Dissociation. To inhibit receptor sequestration, monolayers stably expressing the indicated constructs were pretreated with 100 μM PAO in incubation buffer for 5 minutes at 37°C and then incubated with 2–4 nM [^{3}H]BK on ice for 90 minutes. Thereafter, cells were thoroughly washed with ice-cold PBS and incubated with 1 μM BK in prewarmed incubation buffer at 37°C. At the indicated times, [^{3}H]BK dissociation was stopped by removing the supernatant. Remaining surface-bound [^{3}H]BK was dissociated and determined with ice-cold dissociation solution as described above.

Determination of Receptor Distribution with [^{3}H]JSM10292. Confluent cells in 24 or 48 wells were incubated on ice for 3 hours in incubation buffer with degradation inhibitors containing a 30 nM concentration of the cell membrane-permeant antagonist [^{3}H] JSM10292 (Faussner et al., 2012). Nonspecific binding was determined either with a 1000-fold excess of unlabeled JSM10292 (calculated specific binding comprises intracellular and surface receptors) or BK (calculated specific binding covers only surface receptors). The amount of intracellular receptors is calculated as the difference between specific binding obtained with JSM10292 and that obtained with BK.

Determination of Total Inositol Phosphate Accumulation. Monolayers of stably transfected HEK293 cells on 12 wells were incubated overnight with 0.5 ml complete medium containing 1 μCi/ml [^{3}H]inositol. The cells were washed twice with PBS and preincubated for 90 minutes on ice in incubation buffer supplemented with 50 mM LiCl with or without addition of the indicated concentration(s) of (pseudo)peptide. Stimulation was started by placing the cells in a water bath at 37°C and was continued for 30 minutes. The accumulation of inositol phosphates (IPs) was terminated by exchanging the buffer for 0.75 ml of ice-cold 20 mM formic acid solution. After 30 minutes 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 (Bio-Rad, 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 addition of scintillation liquid.

[^{3}H]BK and [^{3}H]NPC17331 Internalization. [^{3}H] Ligand internalization was determined as described recently (Faussner et al., 2009). In brief, cells on 24-well plates were incubated with 0.2 ml of 2 nM [^{3}H]BK or 2.5 nM [^{3}H]NPC17331 in incubation buffer for 90 minutes on ice to obtain equilibrium binding. [^{3}H] Ligand 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 rinsing the cells with ice-cold PBS. Surface-bound [^{3}H]BK or [^{3}H]NPC17331 was dissociated by incubating the cells for 10 minutes with 0.2 ml of ice-cold dissociation solution. The remaining cell monolayer with internalized [^{3}H] ligand was lysed in 0.2 ml of 0.3 M NaOH and transferred to a scintillation vial. The radioactivity of both samples was determined in a β-counter after addition of scintillation fluid. Non–receptor-mediated [^{3}H] ligand 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 amount of internalized [^{3}H] ligand as a percentage of the combined amounts of internalized and surface bound [^{3}H] ligand.

Biotinylation Protection Assay. Confluent cell monolayers were incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-SS-Biotin (Pierce) in PBS for 30 minutes at 4°C. After rinsing twice with ice-cold Tris-buffered saline (TBS) to quench the biotinylation reaction, cells were equilibrated in Opti-MEM I (Invitrogen) for 30 minutes at 37°C and further incubated in absence or presence of 10 μM bradykinin or 5 μM icatibant. After rinsing with ice-cold TBS, cells were stripped with 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, and 1% FCS for 30 minutes at 4°C, wherever indicated. Glutathione was quenched with 50 mM iodoacetamide and 1% BSA for 20 minutes at 4°C. All cells were washed twice with ice-cold PBS and lysed with extraction buffer (pH 7.4) containing 0.1% Triton X-100, 10 mM Tris-HCl, 150 mM NaCl, 25 mM KCl, and a protease inhibitor cocktail (Complete Mini, EDTA-free; Roche, Mannheim, Germany). Lysates were centrifuged for 15 minutes with 14,000 rpm at 4°C in a microcentrifuge. The supernatant was added to 20 μl of EZview red anti-HA affinity gel pre-equilibrated with extraction buffer and incubated under gentle mixing for 1 hour at 4°C. Subsequently, the matrix was washed extensively with extraction buffer and denatured with NuPAGE LDS sample buffer (Invitrogen) for 5 minutes at 95°C without reducing agents. Proteins were fractionated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes, which were blocked for 1 hour in TBS containing 1% Tween 20 and 5% milk powder. Biotinylated proteins were detected by the Vectastain avidin-biotinylated enzyme complex immunoperoxidase reagent (Vector Laboratories, Burlingame, CA) and developed with Western blot Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences) (Feierler et al., 2011).

Receptor Phosphorylation. Stably transfected cells on 6-well plates were washed twice with phosphate-free DMEM, incubated for 1 hour at 37°C, and labeled with 0.2 mCi/ml [^{32}P]orthophosphate for 1 hour. After 5 minutes of stimulation with 1 μM BK at 37°C, cells were scraped in lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 25 mM KCl, 0.1% Triton X-100] supplemented with a protease inhibitor cocktail (Complete Mini, EDTA-free; Roche, Mannheim, Germany) and centrifuged at 14,000 rpm for 15 minutes at 4°C. Immunoprecipitation of HA-tagged receptor proteins was performed by incubation of the supernatant with 15 μl of EZview red anti-HA affinity gel for 1 hour at 4°C. After washing the matrix in three steps with ice-cold lysis buffer and addition of 30 μl of 1× NuPAGE LDS Sample Buffer (Invitrogen) containing 0.1 M DTT, immunocomplexes were dissociated at 95°C for 10 minutes. Proteins were separated as described above on a 4–12% SDS-PAGE gel. Receptor phosphorylation was detected by autoradiography.

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

Homology Modeling. Sequence alignment of the human B_2R with the bovine rhodopsin performed by ClustalW (Saitou and Nei, 1987; Chenna et al., 2003) showed 19% of identical amino acids and 34% of similar amino acids. In the end of TM3 and the beginning of TM6 with E6.30^{238}, 32% of the residues are identical and 47% are similar. The human B_2R model was obtained by using the homology modeling tool of the Molecular Operating Environment V2010.11 (Chemical Computing Group, Inc.) with the following settings: 10 main chain models each with 3 side chain samples at a temperature of 300 K using the amber99 force field (Wang et al., 2000) were built, resulting in 30 intermediate models with an RMS gradient of 1. Of these 30 models, the final homology model was built by applying refinement protocols with an RMS gradient of 0.5. Subsequently, side chain positions were refined to optimize the protein geometry taking into account typical dihedral angle distributions. Models for mutant B_2R were generated using the mutation tool of Molecular Operating Environment.

Data Analysis. All experiments were performed at least three times in duplicates or triplicates, and results are given as the mean ± S.E.M. unless otherwise indicated. Data analysis was performed using GraphPad Prism for Macintosh, Version 4.0c (GraphPad Software, Inc., San Diego, CA).

Results

To determine the biochemical and functional role of the two conserved residues R3.50 and E6.30 in B_2R regulation,

several receptor mutants were generated: (1) by single mutation of the respective residues in the B_2Rwt, designated R3.50× or E6.30×; (2) by combined charge-neutralizing mutations of both corresponding residues to alanines (R3.50A/E6.30A); and (3) by mutual swapping of the respective amino acids R3.50 and E6.30 (R3.50E/E6.30R). All receptor mutants were stably expressed in HEK293 cells, and their phenotypes were characterized with regard to surface receptor expression, their affinity state at 4°C and 37°C, their capacity to induce PI hydrolysis, basal and ligand-induced internalization, their phosphorylation pattern, and receptor distribution.

Use of Different Promoters to Obtain Comparable Surface Expression. We have shown recently for the B_2Rwt that high overexpression (> 10 pmol/mg protein under the control of the cytomegalovirus promoter) turned ligands, such as icatibant or B9430, known as antagonists in endogenously B_2R expressing cells, into partial agonists. These effects were not observed when the B_2Rwt was expressed at a lower level (<5 pmol/mg protein) as obtained by the P_{min} promoter that consists of only the last 51 nucleotides of the cytomegalovirus promoter (Faussner et al., 2009). To ensure comparable surface expression levels (B_{max}) for wild-type and mutant receptor constructs, mutant receptors with their generally lower surface binding were still expressed under the control of the strong cytomegalovirus promoter. Thus, similar stable expression levels were obtained for all constructs (Table 1).

Single E6.30 Mutants Display High Affinity States at 37°C. Binding studies at 4°C revealed no significant differences between wild-type and mutant receptor constructs with regard to their binding affinity K_d (Table 1). However, unless respective mutations affect directly the binding site, equilibrium binding with [^{3}H]BK at 4°C in our experience always results in comparable high affinity binding for B_2R constructs (Faussner et al., 2009) and, thus, is not really meaningful for determination of the affinity state. With receptor internalization blocked by pretreatment with 100 μM PAO, it is possible to get valuable information on the affinity state of a construct at 37°C in intact cells by determination of the dissociation rate of [^{3}H]BK. A well described example for a constitutively active B_2R mutant in a permanent high affinity state is construct N3.35^{113}A (Marie et al., 1999). Accordingly, the [^{3}H]BK dissociation observed for this mutant was much slower than that found for the B_2Rwt, which shows a strong shift to lower affinity at 37°C (Faussner et al., 2004); after 10 minutes at 37°C, N3.35^{113}A had still more than 60% of the initial [^{3}H]BK bound, whereas B_2Rwt already after 5 minutes had only 5% left (Fig. 2). Mutations of R3.50 resulted in dissociation rates that were comparable to those determined for the B_2Rwt, indicating a low affinity state for these constructs at 37°C. In strong contrast, both single mutations of E6.30 suggested a high affinity state, with E6.30R displaying an even significantly slower [^{3}H]BK dissociation than mutant E6.30A, comparable to that of N3.35^{113}A (Fig. 2). This high affinity state of the E6.30 mutants depended, however, strongly on the presence of R3.50, because both double mutants, R3.50A/E6.30A and R3.50E/E6.30R, displayed fast [^{3}H]BK dissociation, indicating a low-affinity state. These differences between the dissociation rates obtained for the various constructs at 37°C were well supported by the K_d values of the constructs measured at 37°C (Table 1). K_d values were obtained after inhibition of receptor internalization by PAO pretreatment but were less reproducible and, therefore, less reliable for determination of the affinity state than the dissociation data.

Basal and Stimulated PI Hydrolysis. B_2Rwt responded to challenge with 1 μM BK with a 12-fold increase in the accumulation of total IPs, compared with basal levels (Fig. 3A). Substitution of R3.50 with small neutral or negatively charged residues, such as alanine, histidine, or glutamate, abolished PI hydrolysis after BK stimulation, highlighting the crucial role of R3.50 for G protein activation also in the B_2R (Fig. 3A; Table 1). BK stimulation of construct E6.30A, in contrast, resulted in an almost 14-fold higher IP accumulation, compared with basal levels. Because basal phospholipase C β activity was not significantly elevated in E6.30A-expressing cells (Fig. 3A), this construct was not constitutively active with regard to G protein–mediated signal activity, although it displays a high-affinity state at 37°C (Fig. 2 ; Table 1). However, it has been shown that, in addition to constitutively active receptor mutants, there are constructs that display normal basal activity but give strong responses to weak partial agonists and, therefore, are considered to be semi-active (Ballesteros et al., 2001; Fritze et al., 2003). In

TABLE 1
[^{3}H]BK binding data, basal and BK-induced IP accumulation
Results are the mean ± S.E.M. of n independent experiments (numbers in brackets).

Receptor Construct	[^{3}H]BK Binding			Inositol Phosphate Accumulation		
	B_{max}[a]	K_d (4°C)	K_d (37°C)	Basal[b] [X-Fold Over Basal at 4°C]	Maximal Effect[b] [X-Fold Over Basal at 4°C]	EC_{50}[c]
	pmol/mg protein	*nM*				*nM*
B_2Rwt	4.5	2.02 ± 0.22 (5)	8.14 ± 1.19 (8)	1.69 ± 0.09 (4)	12.11 ± 1.22 (4)	0.67 ± 0.22 (3)
R3.50A	3.9	2.09 ± 0.17 (6)	5.53 ± 0.64 (6)	1.30 ± 0.26 (3)	2.12 ± 0.22 (3)	n.a.
R3.50D	4.2	n.d.	n.d.	2.8 (1)	2.4 (1)	n.a.
R3.50E	n.d.	n.d.	7.22 (1)	1.47 ± 0.40 (3)	1.87 ± 0.55 (3)	n.a.
R3.50H	2.4	n.d.	n.d.	1.72 ± 0.32 (4)	1.80 ± 0.29 (4)	n.a.
E6.30A	3.4	1.10 ± 0.19 (5)	2.17 ± 0.39 (5)	2.09 ± 0.02 (3)	13.79 ± 1.66 (3)	2.65 ± 0.73 (4)
E6.30R	4.8	1.00 ± 0.10 (3)	1.44/1.01 (2)	2.33 ± 0.52 (3)	6.03 ± 1.10 (3)	0.64 ± 0.24 (3)
R3.50A/E6.30A	2.4	1.87 ± 0.46 (3)	6.14 ± 1.51 (5)	1.71 ± 0.62 (3)	1.9 ± 0.71 (3)	n.a.
R3.50E/E6.30R	4.5	2.88 ± 0.42 (3)	12.17 ± 0.64 (3)	1.61 ± 0.36 (3)	3.44 ± 1.15 (3)	n.a.

n.a., not applicable; n.d., not determined.
[a] Estimated with approximately 30 nM [^{3}H]BK at 4°C.
[b] Total IP accumulation after 30 minutes of incubation in buffer with inhibitors and 50 mM LiCl at 37°C without (basal) and with (maximal effect) 1 μM BK, expressed as fold increase of initial total IP content (t=0 min).
[c] Calculated from incubations in duplicates with 10^{-12}-10^{-5} M BK for 30 minutes at 37°C in the presence of 50 mM LiCl.

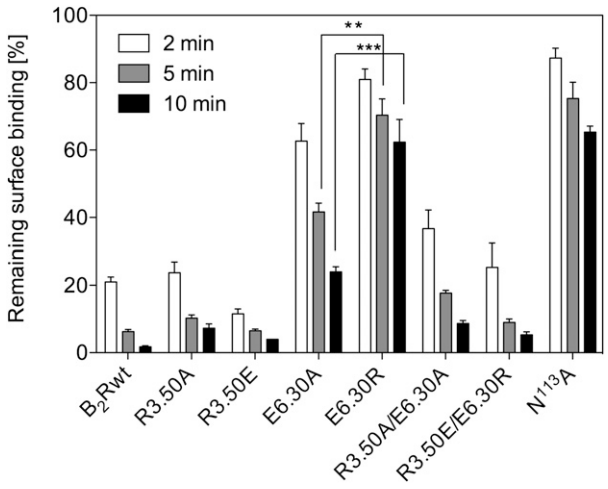


Fig. 2. [^{3}H]BK dissociation. After pretreatment with phenylarsine oxide, cells stably expressing the indicated constructs were incubated with 2–4 nM [^{3}H]BK on ice for 90 minutes. Subsequently, cells were rinsed at 4°C and incubated with 1 μM BK in incubation buffer at 37°C. At the indicated times, [^{3}H]BK dissociation was stopped and remaining surface bound [^{3}H]BK was determined as described in *Materials and Methods*. Data are shown as percentage of initial binding at 4°C. Columns represent the mean ± S.E.M. (n = 3–11). (One-way ANOVA with Newman-Keuls Multiple Comparison Test: $^{**}P < 0.001$; $^{***}P < 0.001$).

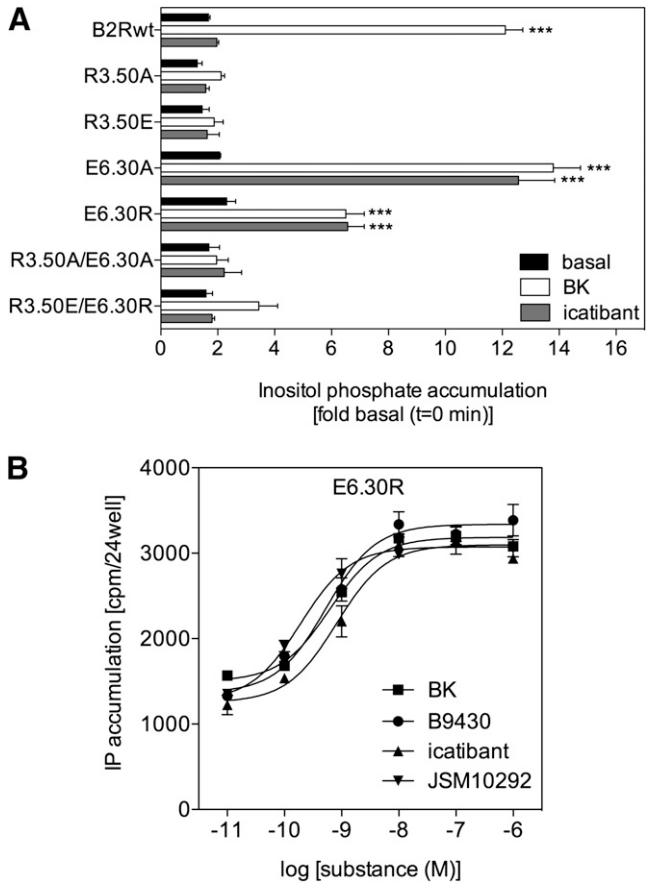


Fig. 3. IP accumulation. (A) basal and stimulated IP accumulation. Cells expressing the indicated receptor constructs were preincubated overnight with [^{3}H]inositol. IP accumulation in the presence of 50 mM LiCl after incubation for 30 minutes at 37°C with 1 μM BK or icatibant was determined as described in *Materials and Methods*. Each column represents the mean ± S.E.M. (n = 3–7). The results are presented as fold increase over the IP content of identically treated control cells at 4°C. (One-way ANOVA with Newman-Keuls Multiple Comparison Test: $^{***}P < 0.001$). (B) dose-dependent IP accumulation of mutant E6.30R-expressing cells in response to B_2R agonist and antagonists. HEK293 cells stably expressing mutant E6.30R and preincubated overnight with [^{3}H]inositol were stimulated with the indicated concentrations of BK, B9430, icatibant, or JSM10292 for 30 minutes at 37°C. Shown is a representative experiment that was repeated three times with similar results.

line with the idea of semi-activity, we have shown previously that some B_2R mutants respond with robust PI hydrolysis to the antagonists icatibant and B9430 (Faussner et al., 2009). As depicted in Fig. 3A, in E6.30A- and E6.30R-expressing cells, icatibant displayed the same efficacy as BK, suggesting semi-active conformations for these mutants. As demonstrated by the dose-response curves shown in Fig. 3B, all the antagonists, B9430, icatibant, and even the small molecule antagonist JSM10292 (Gibson et al., 2009), stimulated mutant E6.30R with practically identical potency and efficacy as BK. In cells expressing mutant E6.30A, all the tested antagonists (icatibant and JSM10292) also behaved as agonists, however, with slightly lower potency, compared with the stimulation observed with the agonist BK (Supplemental Fig. 1). Thus, mutation of E6.30 to an arginine or to an alanine turned all the tested B_2R ligands into strong agonists. In contrast, mutation of the two amino acids and potential interaction partners of R3.50 in the DRY motif, D3.49, or Y3.51, to alanines, did not change BK-induced PI hydrolysis , compared with B_2Rwt (data not shown). Unsurprisingly, mutant R3.50A, which did not respond to BK stimulation, was incapable of generating an IP signal after challenge with the antagonist icatibant, and double substitution of R3.50 and E6.30 with alanines created the same phenotype as the single R3.50A mutation (Fig. 3A). Of interest, mutual swapping of R3.50 and E6.30 (R3.50E/E6.30R) induced a clear tendency to BK-stimulated PI hydrolysis, which, however, did not turn significant (Fig. 3A).

Negative Charge at Position 3.50 Disturbs B_2R Internalization. After stimulation with BK, the B_2R gets rapidly internalized (Leeb-Lundberg et al., 2005). To functionally characterize the effects of disruption of the ionic lock by mutating either R3.50 or E6.30, changes in [^{3}H]BK uptake were determined over a short period of 30 minutes, for which a similar fate of receptor and ligand can be assumed.

After 10 minutes, B_2Rwt-expressing cells had internalized almost 90% of the specifically bound [^{3}H]BK to acetic acid–resistant compartments (Fig. 4A). Charge-neutralizing mutation of R3.50 to alanine had no significant negative effect, indicating that G protein activation is not a prerequisite for [^{3}H]BK internalization. Only replacement of the positively charged R3.50 with negatively charged residues, such as glutamate or aspartate, significantly reduced the internalization rate (Fig. 4A). This was not a direct effect of a negatively charged amino acid in position 3.50 on the internalization mechanism per se, because fast internalization could be rescued by an additional mutation of either D3.49 to an arginine or E6.30 to an alanine or an arginine (Fig. 4C). Accordingly, the respective single mutations of E6.30 also had no effect on [^{3}H]BK internalization (Fig. 4B).

R3.50A and E6.30A/R Internalize Antagonist [^{3}H]NPC17331. Charge neutralization or charge reversal of E6.30 resulted in strong PI hydrolysis even in response to B_2R antagonists (Fig. 3), indicating a semi-active conformation of these mutants with regard to G protein interaction.

Our next goals were to determine (1) whether this was limited to G protein activation or also the case with regard to receptor internalization and (2) whether this was attributable to interruption of the ionic lock in general or only caused by the point mutation of E6.30.

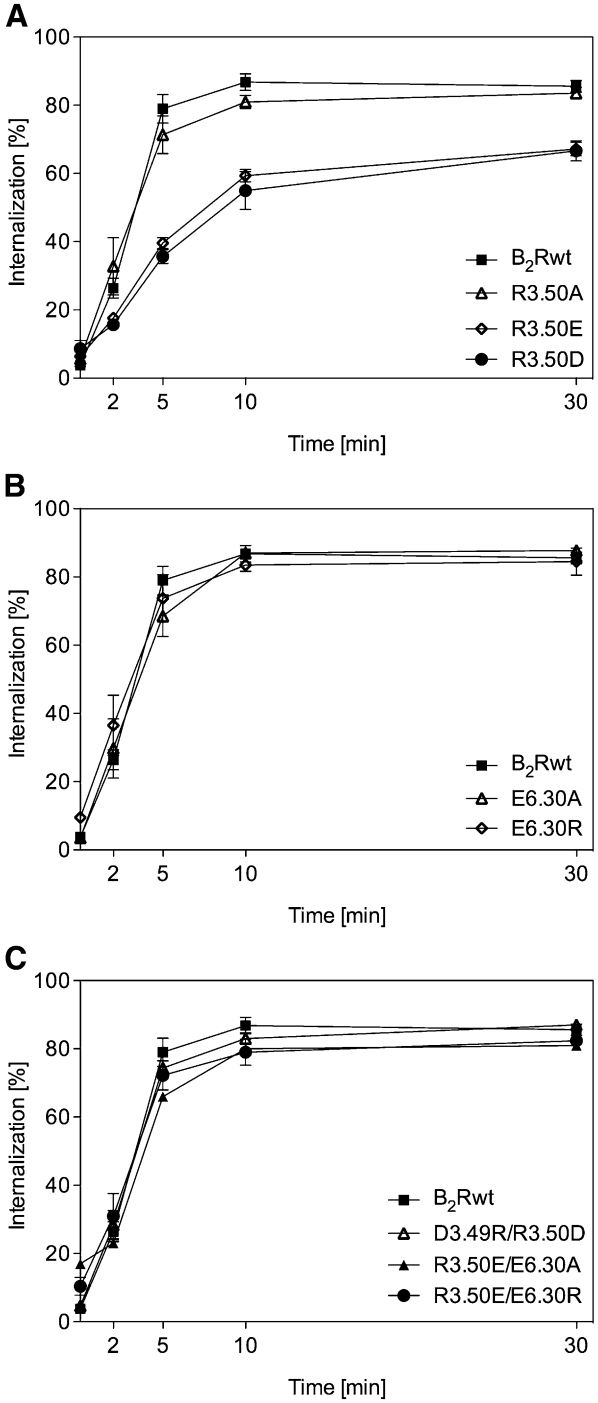


Fig. 4. Internalization of [^{3}H]BK. Cells expressing B_2Rwt, R3.50× (X=A, E,D) and E6.30× (X=A,E) were preincubated with 2 nM [^{3}H]BK for 90 minutes on ice. Internalization was started by warming the plates to 37°C. After the indicated times, surface-bound and internalized [^{3}H]BK were determined by acetic acid treatment as described in *Materials and Methods*. Internalization is given as percentage of total bound [^{3}H]BK (surface plus internalized [^{3}H]BK). Points represent means ± S.E.M. (n = 3–5). Curves of R3.50E and R3.50D were significantly different from B_2Rwt and R3.50A. (*$P < 0.05$; one-way ANOVA with Newman-Keuls Multiple Comparison Test).

In contrast to B_2Rwt, which did not internalize the antagonist [^{3}H]NPC17331 (about 10% internalization after 10 minutes at 37°C), in R3.50A mutant cells, about 25% of specifically bound [^{3}H]NPC17331 were internalized after 10 minutes at 37°C (Fig. 5). Mutant receptors E6.30A and E6.30R showed even stronger antagonist internalization (~60% within 10 minutes). Simultaneous mutation of both residues R3.50 and E6.30 to alanines created receptors strongly internalizing [^{3}H]NPC17331 comparable to mutant E6.30A. In contrast, double mutation by mutual swapping of R3.50 and E6.30 restored the phenotype of B_2Rwt, because mutant R3.50E/E6.30R only slightly internalized [^{3}H] NPC17331 (~17% internalization after 10 minutes at 37°C). The results show that abolishing ionic lock formation by mutating either of the interacting residues results in increased internalization of the antagonist [^{3}H]NPC17331.

Biotinylation Protection Assay Reveals Constitutive Internalization. The observed uptake of [^{3}H]NPC17331 could either be attributable to ligand-induced receptor internalization (partial agonist effect) or result from ligand-independent constitutive receptor internalization. To distinguish between these two possibilities, a biotinylation protection assay was performed (Feierler et al., 2011). Cell surface receptors were labeled at 4°C with a membrane-impermeable biotin derivative containing a disulfide linker. Receptors that had been internalized thereafter at 37°C in a ligand-dependent or independent manner were selectively identified by immunoprecipitation and Western blot analysis. Because of their localization within intracellular compartments as a consequence of endocytosis, their biotin label would have been protected from cleavage by the extracellular treatment with reducing glutathione.

B_2Rwt was prominently internalized only after BK stimulation (Fig. 6), demonstrating that B_2Rwt internalization is strictly dependent on agonist stimulation. Breaking the ionic

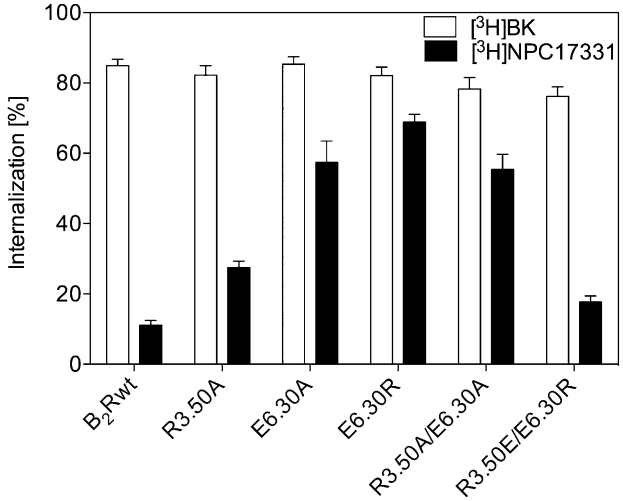


Fig. 5. Comparison of [^{3}H]NPC17331 and [^{3}H]BK internalization. HEK293 cells stably expressing the indicated constructs were preincubated with 2 nM [^{3}H]BK or [^{3}H]NPC17331 for 90 minutes on ice. Internalization was induced by warming the plates to 37°C. After 10 minutes, surface-bound and internalized [^{3}H]BK or [^{3}H]NPC17331 were determined by acetic acid treatment as described in *Materials and Methods*. Internalization is presented as percentage of total bound [^{3}H] ligand (surface plus internalized [^{3}H]BK or [^{3}H]NPC17331). Columns represent means ± S.E.M. of three experiments performed in triplicate.

lock by mutating the single residues to alanines (R3.50A, E6.30A) generated receptors that internalized from the cell surface in a constitutive manner (i.e., in the absence of any agonist). Addition of BK augmented this internalization (significantly for R3.50A, as a tendency only for E6.30A) to a similar level as observed for the B_2Rwt. Simultaneous mutation of R3.50 and E6.30 to alanines (R3.50A/E6.30A) resulted in a construct showing maximal constitutive internalization that could no longer be increased by addition of BK. Intriguingly, any ionic lock between position 3.50 and position 6.30 is sufficient to keep B_2R internalization fully agonist dependent, because mutually swapping both residues (R3.50E/E6.30R) completely abolished ligand-independent, constitutive internalization. With regard to receptor internalization, icatibant behaved as a neutral antagonist, because in all cases, it neither enhanced nor inhibited (constitutive) receptor internalization (Fig. 6).

E6.30 Mutation Significantly Increases Basal Receptor Phosphorylation. According to the widely accepted model of GPCR trafficking, receptor phosphorylation mostly by GPCR kinases (GRKs) is considered to be a major requirement for internalization. Thus, we next investigated whether the ligand-dependent and independent internalization behavior of the various B_2R constructs is also reflected in their respective phosphorylation patterns.

The B_2Rwt was basally phosphorylated and reacted to BK stimulation with an ~3-fold increase in phosphorylation intensity (Fig. 7), in agreement with previous publications (Blaukat et al., 1996; Blaukat et al., 2001). Mutant R3.50A showed a slightly increased basal phosphorylation and was BK sensitive. Compared with BK-induced B_2Rwt phosphorylation, however, the level of agonist-stimulated R3.50A phosphorylation was almost 40% significantly lower ($P < 0.05$) (Fig. 7B). Charge-neutralizing substitution of E6.30 with alanine significantly intensified basal receptor phosphorylation by almost 30%, compared with the B_2Rwt; however, BK treatment further increased phosphorylation. In contrast, charge-neutralizing mutation of both residues (R3.50A/E6.30A) resulted in the strongest basal phosphorylation observed for all constructs, which could also no longer be significantly augmented by addition of BK. Mutual swapping of R3.50 and E6.30 (R3.50E/E6.30R; i.e., generation of an inverse ionic lock) almost completely reconstituted the B_2Rwt-like phosphorylation pattern with lower basal phosphorylation and strong additional phosphorylation after stimulation with BK.

When analyzing receptor phosphorylation (Fig. 7), the B_2Rwt was also expressed under the control of the strong cytomegalovirus promoter, resulting in elevated expression levels of ~10 pmol/mg protein. Comparing receptor expression levels from whole cell lysates after immunoprecipitation (Fig. 7A), no remarkable differences between B_2Rwt (with 10 pmol) and mutant constructs were detected, conversely to the results obtained from [^{3}H]BK binding experiments, which indicated much lower (cell surface) expression levels for the mutant receptor constructs (Table 1). This discrepancy between surface binding data and results from Western blot analysis suggested that mutant receptor constructs might also be located intracellularly (e.g., as a result of constitutive internalization).

Double Mutant R3.50A/E6.30A Is Largely Located Intracellularly. We previously characterized a novel cell membrane-permeant small molecule, JSM10292, which, ^{3}H-labeled, allows the differentiation between surface and intracellularly located wild-type and mutant B_2Rs, as long as they are binding competent (Faussner et al., 2012). Unlike the B_2Rwt, which, unstimulated, is located mostly at the cell surface (~80%), >50% of the single mutant receptor constructs R3.50A and E6.30A were found to be located intracellularly (Table 2). Strikingly, double mutation of both residues to alanines (R3.50A/E6.30A) lead to a strong intracellular localization of about 70%, whereas mutual exchange of both highly conserved residues (R3.50E/E6.30R) generated a wild-type–like surface localization (Table 2).

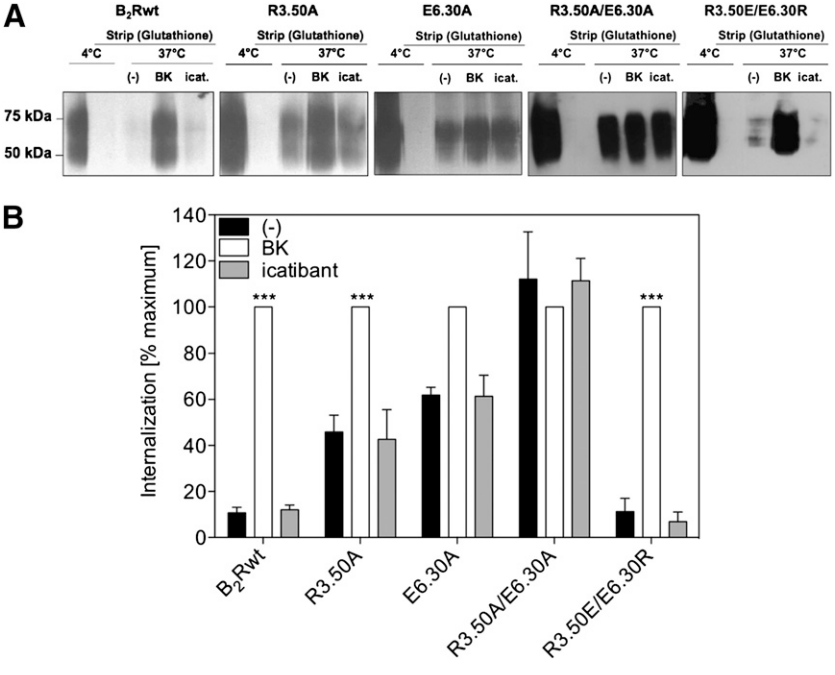


Fig. 6. Receptor internalization determined by biotinylation protection assay. (A) HEK293 cells stably expressing the indicated N-terminally HA-tagged constructs were labeled at 4°C with biotin reagent containing a reducible disulfide linker. Cells were either stripped directly with 50 mM glutathione at 4°C (Strip), or incubated in the absence (−) or presence of 10 μM BK or 5 μM icatibant (icat.) for 1 hour at 37°C and then stripped at 4°C. Immunoprecipitation of receptors and detection of their biotinylation status by Western blot analysis was performed as described in *Materials and Methods*. The blots shown are representative for three experiments. (B) biotinylated receptors were quantified with ImageJ as described in *Materials and Methods* and normalized to the amount of biotinylated receptors obtained after BK stimulation that served as a reference for maximal response (= 100%). (One-way ANOVA with Newman-Keuls Multiple Comparison Test: ***$P <$ 0.001).

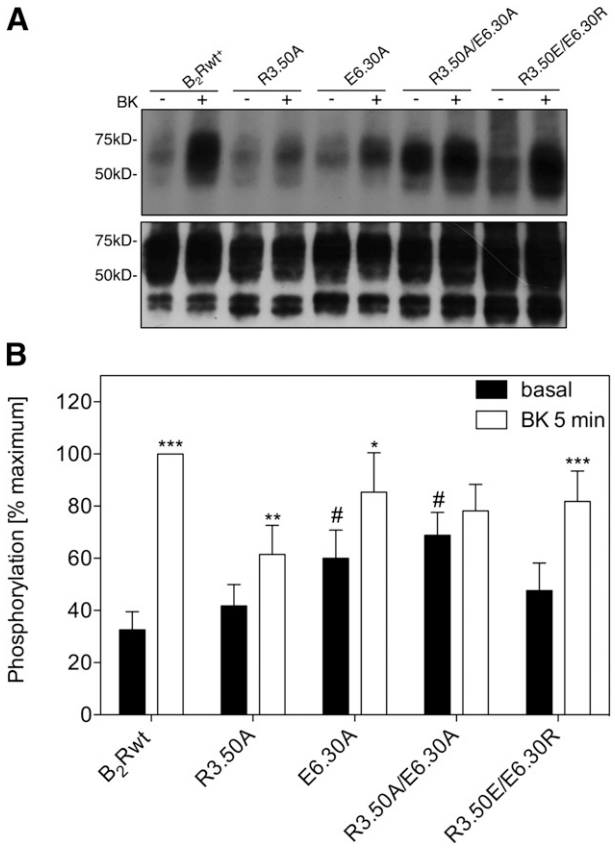


Fig. 7. Receptor phosphorylation. (A) receptor-expressing cells on 6-well plates were labeled with [^{32}P]orthophosphate and stimulated with 1 μM BK for 5 minutes at 37°C. HA-tagged receptors were immunoprecipitated as described in *Materials and Methods* and separated by SDS-PAGE. Receptor phosphorylation was detected by autoradiography (top), and receptor expression levels (bottom) are shown as a control. The blot depicted is representative (n = 4–5). (B) amounts of phosphorylated receptors were quantified with ImageJ as described in *Materials and Methods* and normalized to BK-stimulated B_2Rwt that served as positive control and reference for maximum phosphorylation (= 100%). (t test: basal versus BK (5 minutes): ***$P < 0.001$, ** $P < 0.01$, * $P < 0.1$; basal [B_2Rwt] vs. basal [receptor mutants]: $^{\#}$ $P < 0.1$).

Discussion

Homology modeling of the B_2R, based on the bovine rhodopsin structure as a template, displayed a salt bridge between R3.50 and E6.30 that connects TM3 with TM6 (Fig. 8A), thus stabilizing the inactive conformation. An additional network of hydrogen bonds around R3.50 and E6.30, involving the side chains of D3.49 and T6.34 and the carbonyl oxygens of A6.33 and E6.30, supports the ionic lock in this B_2R model. All these residues can be found in identical positions in many other family A GPCRs, suggesting similar structural networks.

Function of the DRY Motif with R3.50

In the B_2R, a single substitution of R3.50 with other amino acids (A, H, D, E) completely abolished the receptor's ability to induce PI hydrolysis (Table 1), arachidonic acid release (shown for R3.50A; Supplemental Fig. 2), and ERK1/2 phosphorylation (Supplemental Fig. 3). This emphasizes the significance of this residue for productive G protein interaction, which has also been observed for almost all other family A GPCRs investigated thus far (Scheer et al., 1996; Ballesteros et al., 2001).

TABLE 2

Receptor distribution

HEK293 cells expressing the indicated construct were incubated for 3 hours at 4°C with 30 nM of the cell membrane-permeant B_2R antagonist [^{3}H]JSM10292 in the absence or presence of either 30 μM unlabeled JSM10292 (specific surface and intracellular binding) or 30 μM BK (specific surface binding only). The amount of intracellular receptor binding is given as mean ± S.E.M. and as percentage of total receptor binding (intracellular plus surface). The number of independent experiments performed in triplicates is given in parentheses.

Receptor construct	Intracellularly Located Receptors
	% of total
B_2Rwt	18 ± 3 (7)
R3.50A	64 ± 6 (3)
E6.30A	57 ± 6 (3)
R3.50A/E6.30A	71 ± 4 (4)
R3.50E/E6.30R	24 ± 7 (5)

For GPCRs that become phosphorylated by GRK2/3 during their desensitization, it has been proposed that these GRKs are recruited by attaching with their pleckstrin homology domains to $\beta\gamma$-subunits that become available after G protein activation (Willets et al., 2003). This mechanism is obviously not the only plausible one, because all signaling-incompetent R3.50 mutants were phosphorylated (shown for R3.50A; Fig. 7) and internalized similarly to the B_2Rwt (Fig. 4). Fast internalization has also been reported for a G protein activation-incompetent R3.50A mutant of the type 1 angiotensin receptor (AT_1R) (Gaborik et al., 2003). Disruption of the ionic bond might therefore result in a conformation that directly interacts with GRK2/3. Such a direct interaction that was primarily dependent on an intact helix 8 of the receptor has been demonstrated for wild-type and truncated B_2R constructs (Feierler et al., 2011).

GPCRs can activate MAPK cascades G protein dependently and/or via the recruitment of β-arrestins (Shenoy and Lefkowitz, 2005). Our data support a predominantly G protein–dependent MAPK activation by the B_2R, because the PI hydrolysis-incompetent mutant R3.50A becomes internalized rapidly after BK stimulation (Fig. 4) and, therefore, should interact with β-arrestins (as also indicated by translocation studies with eYFP-β-arrestin 1 and 2; data not shown) but, nevertheless, does not induce ERK1/2 phosphorylation (Supplemental Fig. 3). In contrast, the AT_1R DRY mutant was still able to elicit ERK1/2 phosphorylation in a β-arrestin–dependent way, as demonstrated by siRNA-knockdown experiments (Wei et al., 2003). Thus, whether an interaction with β-arrestins results not only in internalizaltion but also in ERK1/2 activation might depend on additional factors (e.g., a receptor-specific GRK-generated phosphorylation pattern) (Nobles et al., 2011).

Although for other GPCRs, residue D3.49 was reported to play an important role in G protein–dependent signaling (Scheer et al., 1996; Rasmussen et al., 1999), we did not observe any significant differences between mutant D3.49A and the B_2Rwt with regard to PI hydrolysis, ligand-mediated internalization, or affinity state at 37°C (data not shown). This stresses the importance of the structural context of these highly conserved residues, because they apparently not do play necessarily the same roles in different family A GPCRs.

Function of E6.30 in the Activation Process

Charge neutralization or reversal of E6.30 resulted in B_2R constructs that responded with strong PI hydrolysis and

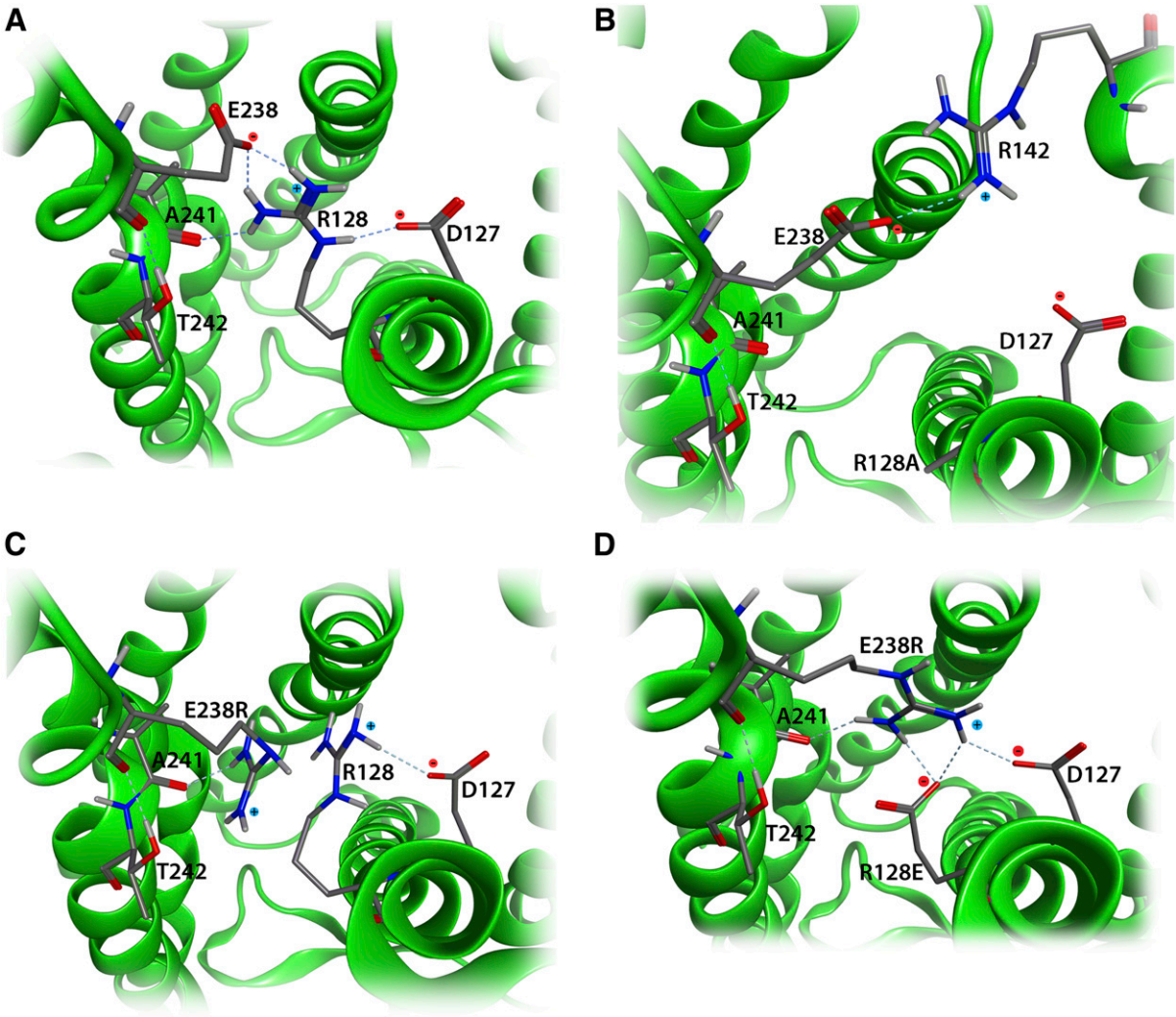


Fig. 8. Homology model of wild-type and mutant B_2R constructs. (A) B_2Rwt: salt bridge between R3.50^{128} and E6.30^{238} termed ionic lock and hydrogen-bonding network, including side chains of D3.49^{127} and T6.34^{242} and carbonly oxygens of A6.33^{241} and E6.30^{238}. (B) mutant R3.50A: disruption of the ionic lock might result in interaction of E6.30^{238} with Arg142 through a newly formed salt bridge. (C) mutant E6.30R: mutation might generate repulsion due to identical charges between TM6 with E6.30^{238}R and TM3 with R3.50^{128}. (D) mutant R3.50E/E6.30R: Double mutation allows for formation of an inverse ionic lock connecting TM3 (R3.50^{128}E) and TM6 (E6.30^{238}R).

ERK1/2 phosphorylation (Supplemental Fig. 3; not shown for E6.30R) not only to the agonist BK, but also to the antagonists B9430, icatibant, or JSM10292 (Fig. 3; Supplemental Fig. 1). Contrary to the β_2-adrenergic receptor, where E6.30A mutation elevated basal receptor activity (Ballesteros et al., 2001), or the 5-hydroxytryptamine 2A (5-HT2A) receptor, in which E6.30R mutation evoked a high constitutive activity (Shapiro et al., 2002), the analog B_2R mutants showed no or only minor increases in basal IP generation (Fig. 3) or MAPK stimulation (Supplemental Fig. 3). However, they apparently adapted a highly sensitive conformation, as also indicated by their high affinity state at 37°C (Fig. 2; Table 1), that could be easily activated by binding of all kinds of ligands. The data also demonstrate that a high affinity state of a GPCR does not necessarily imply constitutive activity. Similarly, E6.30 mutations in the thromboxane prostanoid receptor resulted in more efficient agonist-induced signaling without any increase in basal activity (Ambrosio et al., 2010). Moreover, for the B_2R, even the small molecule compound JSM10292, which thus far had displayed no partial agonistic activity (Faussner et al., 2012), becomes a full agonist in mutant E6.30R. This might be explained by assuming a semi-active conformation due to repulsion of the two positively charged arginines (Fig. 8C) that imitates in a way a structural change—TM6 moving away from TM3—that has been described as part of the activation process (Ballesteros et al., 2001; Springael et al., 2007; Rasmussen et al., 2011). In addition, mutation E6.30A/R resulted in strong uptake of the antagonist [^{3}H] NPC17331, increased basal phosphorylation, constitutive internalization, and consequently, considerable intracellular localization. These data strengthen the idea that the E6.30 mutants adopt a conformation that constitutively interacts with GRKs and β-arrestins, but not with G proteins, because they display no significant agonist-independent PI hydrolysis.

Is There an Ionic Lock in the B_2R?. Although homology modeling indicated an ionic lock (Fig. 8A) in the B_2R, its presence would set the B_2R apart from other peptide GPCRs, because most of them (>90%) cannot form an ionic lock because of the lack of an acidic residue in position 6.30 (Mirzadegan et al., 2003). Without R3.50 and E6.30 actually

interacting, effects of single mutations should be rather different and, in most cases, additive in the double mutant. In contrast, if they form an ionic lock, the effects of the single mutations, in theory, should give identical results. Both mutants R3.50A and E6.30A internalized the antagonist [^{3}H] NPC17331, both indicated constitutive internalization that could be increased by BK but not icatibant, and both showed elevated basal phosphorylation and similar intracellular localization. Because the double mutation R3.50A/E6.30A resulted in effects that were at least as strong as those of the single mutations, but not significantly higher or additive, the formation of an ionic lock in the B_2R in its inactive state is feasible. The fact that all the observed effects were stronger in the E6.30 mutants than in mutant R3.50A might be explained by residue Arg142 in the second intracellular loop acting as a compensatory interaction partner for E6.30 (Fig. 8B). The strongest indication for an ionic bond in the B_2R is the rescue of wild-type behavior by swapping the respective arginine and glutamate (R3.50E/E6.30R), thus generating an inverse ionic lock (Fig. 8D). 5-HT2A receptor activation seems to follow a similar pattern, because analog swapping of residues 3.50 and 6.30 abolished an increase in basal receptor signaling activity (Shapiro et al., 2002). With regard to basal phosphorylation and constitutive internalization, however, a clear trend toward an additive effect was seen in the double mutant (e.g., the intracellular localization), suggesting that both residues function also via other interaction partners outside the ionic bond. The importance of the respective microenvironment of R3.50 and E6.30 is also indicated by a report that the constitutive activity of the human histamine H_4 receptor, which contains an alanine in position 6.30, cannot be simply reduced by reconstituting an ionic lock generating a mutant A6.30E (Schneider et al., 2010).

Conclusions

Our results indicate that, in the inactive B_2R, R3.50 and E6.30 form an ionic lock. We show for the first time that the ionic lock in a family A GPCR can play different roles when comparing G protein activation with the interaction with GRKs and arrestins in the process of receptor internalization. Its disruption in the B_2R resulted in constitutive internalization with consequently strong intracellular localization of the constructs, but did not change the character of the tested B_2R antagonists in this regard. In contrast, mutation of E6.30 did not result in constitutive G protein activation but turned all tested B_2R antagonists into strong agonists. This suggests a multistep process of B_2R activation, in which the disruption of the salt bridge between R3.50 and E6.30 plays a pivotal but differential role with regard to the different processes of G protein activation and receptor internalization. Thus, our findings strengthen current concepts of biased agonism and functional selectivity. Different ligand-induced conformational changes and subsequent signaling has also been suggested for several GPCRs (Ahn et al., 2004; Kobilka and Deupi, 2007; Rosenbaum et al., 2009), highlighting the structural flexibility of GPCRs and the dynamic nature of their activation process. Similarly, multiple structurally different ligands are known to stabilize distinct conformational states of the β_2-adrenergic receptor, eliciting differential cellular responses (Bhattacharya et al., 2008; Kahsai et al., 2011). Deeper insight into the multistep mechanism of GPCR activation, as provided by our study, and the generation of differentially active mutants might help in the development and screening of new specific biased agonists and antagonists for optimized therapeutic intervention.

Acknowledgments

The authors thank C. Seidl and H. Grondinger for excellent technical assistance.

Authorship Contributions

Participated in research design: Leschner, Wennerberg, , Faussner.

Conducted Experiments: Leschner, Wennerberg, Feierler, Welte, Kalatskaya, Faussner.

Contributed new reagents or analytic tools: Wolber.

Performed data analysis: Leschner, Wennerberg, Feierler, Bermudez, Welte, Kalatskaya, Wolber, Faussner.

Wrote or contributed to the writing of the manuscript: Leschner, Wennerberg, Wolber, Faussner.

References

Ahn S, Wei H, Garrison TR, and Lefkowitz RJ (2004) Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *J Biol Chem* **279**:7807–7811.

Ambrosio M, Fanelli F, Brocchetti S, Raimondi F, Mauri M, Rovati GE, and Capra V (2010) Superactive mutants of thromboxane prostanoid receptor: functional and computational analysis of an active form alternative to constitutively active mutants. *Cell Mol Life Sci* **67**:2979–2989.

Ballesteros J, Kitanovic S, and Guarnieri F, et al. (1998) Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J Biol Chem* **273**:10445–10453.

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U, and Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* **276**:29171–29177.

Bhattacharya S, Hall SE, Li H, and Vaidehi N (2008) Ligand-stabilized conformational states of human beta(2) adrenergic receptor: insight into G-protein-coupled receptor activation. *Biophys J* **94**:2027–2042.

Blaukat A, Alla SA, Lohse MJ, and Müller-Esterl W (1996) Ligand-induced phosphorylation/dephosphorylation of the endogenous bradykinin B2 receptor from human fibroblasts. *J Biol Chem* **271**:32366–32374.

Blaukat A, Barac A, Cross MJ, Offermanns S, and Dikic I (2000) G protein-coupled receptor-mediated mitogen-activated protein kinase activation through co-operation of Galpha(q) and Galpha(i) signals. *Mol Cell Biol* **20**:6837–6848.

Blaukat A, Pizard A, Breit A, Wernstedt C, Alhenc-Gelas F, Müller-Esterl W, and Dikic I (2001) Determination of bradykinin B2 receptor in vivo phosphorylation sites and their role in receptor function. *J Biol Chem* **276**:40431–40440.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, and Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**:3497–3500.

Faussner A, Schuessler S, Seidl C, and Jochum M (2004) Inhibition of sequestration of human B2 bradykinin receptor by phenylarsine oxide or sucrose allows determination of a receptor affinity shift and ligand dissociation in intact cells. *Biol Chem* **385**:835–843.

Faussner A, Schüssler S, Feierler J, Bermudez M, Pfeifer J, Schnatbaum K, Tradler T, Jochum M, Wolber G and Gibson C (2012) Binding characteristics of [(3) H] JSM10292: a new cell membrane-permeant nonpeptide bradykinin B(2) receptor antagonist. *Br J Pharmacol*.

Faussner A, Wennerberg G, Schüssler S, Feierler J, Seidl C, Jochum M, and Proud D (2009) Alanine screening of the intracellular loops of the human bradykinin B receptor—effects on receptor maintenance, G protein activation and internalization. *FEBS J* **276**:3491–3503.

Feierler J, Wirth M, Welte B, Schüssler S, Jochum M, and Faussner A (2011) Helix 8 plays a crucial role in bradykinin B(2) receptor trafficking and signaling. *J Biol Chem* **286**:43282–43293.

Fritze O, Filipek S, Kuksa V, Palczewski K, Hofmann KP, and Ernst OP (2003) Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation. *Proc Natl Acad Sci USA* **100**:2290–2295.

Gáborik Z, Jagadeesh G, Zhang M, Spät A, Catt KJ, and Hunyady L (2003) The role of a conserved region of the second intracellular loop in AT1 angiotensin receptor activation and signaling. *Endocrinology* **144**:2220–2228.

Gibson C, Schnatbaum K, Pfeifer JR, Locardi E, Paschke M, Reimer U, Richter U, Scharn D, Faussner A, and Tradler T (2009) Novel small molecule bradykinin B2 receptor antagonists. *J Med Chem* **52**:4370–4379.

Hess JF, Borkowski JA, Young GS, Strader CD, and Ransom RW (1992) Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem Biophys Res Commun* **184**:260–268.

Jacoby E, Bouhelal R, Gerspacher M, and Seuwen K (2006) The 7 TM G-protein-coupled receptor target family. *ChemMedChem* **1**:761–782.

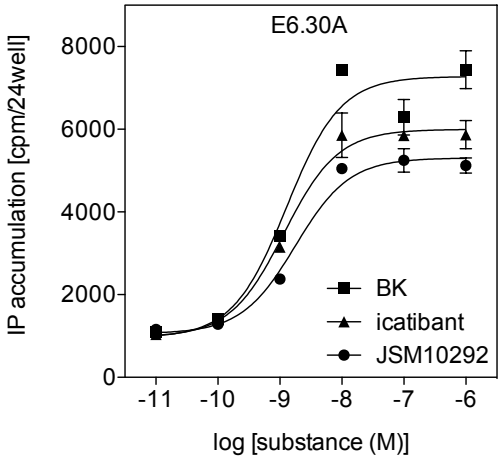
Kahsai AW, Xiao K, Rajagopal S, Ahn S, Shukla AK, Sun J, Oas TG, and Lefkowitz RJ (2011) Multiple ligand-specific conformations of the β2-adrenergic receptor. *Nat Chem Biol* **7**:692–700.

Kobilka BK and Deupi X (2007) Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci* **28**:397–406.
Leeb-Lundberg LM, Marceau F, Müller-Esterl W, Pettibone DJ, and Zuraw BL (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* **57**:27–77.
Leschner J, Ring L, Feierler J, Dinkel K, Jochum M, and Faussner A (2011) Fever-like temperature modification differentially affects in vitro signaling of bradykinin B(1) and B(2) receptors. *Biol Chem* **392**:1021–1029.
Marie J, Koch C, Pruneau D, Paquet JL, Groblewski T, Larguier R, Lombard C, Deslauriers B, Maigret B, and Bonnafous JC (1999) Constitutive activation of the human bradykinin B2 receptor induced by mutations in transmembrane helices III and VI. *Mol Pharmacol* **55**:92–101.
Mirzadegan T, Benkö G, Filipek S, and Palczewski K (2003) Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry* **42**: 2759–2767.
Nobles KN, Xiao K, and Ahn S, et al. (2011) Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci Signal* **4**:ra51.
Nygaard R, Frimurer TM, Holst B, Rosenkilde MM, and Schwartz TW (2009) Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci* **30**: 249–259.
Okada T, Sugihara M, Bondar AN, Elstner M, Entel P, and Buss V (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure. *J Mol Biol* **342**:571–583.
Overington JP, Al-Lazikani B, and Hopkins AL (2006) How many drug targets are there? *Nat Rev Drug Discov* **5**:993–996.
Palczewski K, Kumasaka T, and Hori T, et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739–745.
Rasmussen SG, Choi HJ, and Fung JJ, et al. (2011) Structure of a nanobody-stabilized active state of the β(2) adrenoceptor. *Nature* **469**:175–180.
Rasmussen SG, Jensen AD, Liapakis G, Ghanouni P, Javitch JA, and Gether U (1999) Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol Pharmacol* **56**:175–184.
Rosenbaum DM, Rasmussen SG, and Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature* **459**:356–363.
Rovati GE, Capra V, and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71**: 959–964.
Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**:406–425.
Salon JA, Lodowski DT, and Palczewski K (2011) The significance of G protein-coupled receptor crystallography for drug discovery. *Pharmacol Rev* **63**:901–937.
Scheer A, Fanelli F, Costa T, De Benedetti PG, and Cotecchia S (1996) Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *EMBO J* **15**:3566–3578.
Schneider EH, Schnell D, Strasser A, Dove S, and Seifert R (2010) Impact of the DRY motif and the missing "ionic lock" on constitutive activity and G-protein coupling of the human histamine H4 receptor. *J Pharmacol Exp Ther* **333**: 382–392.
Shapiro DA, Kristiansen K, Weiner DM, Kroeze WK, and Roth BL (2002) Evidence for a model of agonist-induced activation of 5-hydroxytryptamine 2A serotonin receptors that involves the disruption of a strong ionic interaction between helices 3 and 6. *J Biol Chem* **277**:11441–11449.
Shenoy SK and Lefkowitz RJ (2005) Seven-transmembrane receptor signaling through beta-arrestin. *Sci STKE* **2005**:cm10.
Shukla AK, Xiao K, and Lefkowitz RJ (2011) Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem Sci* **36**: 457–469.
Springael JY, de Poorter C, Deupi X, Van Durme J, Pardo L, and Parmentier M (2007) The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin. *Cell Signal* **19**:1446–1456.
Teller DC, Okada T, Behnke CA, Palczewski K, and Stenkamp RE (2001) Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40**:7761–7772.
Vassilatis DK, Hohmann JG, and Zeng H, et al. (2003) The G protein-coupled receptor repertoires of human and mouse. *Proc Natl Acad Sci USA* **100**: 4903–4908.
Vogel R, Mahalingam M, Lüdeke S, Huber T, Siebert F, and Sakmar TP (2008) Functional role of the "ionic lock"—an interhelical hydrogen-bond network in family A heptahelical receptors. *J Mol Biol* **380**:648–655.
Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* **100**:10782–10787.
Wang JM, Cieplak P, and Kollman PA (2000) How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? *J Comput Chem* **21**:1049–1074.
Willets JM, Challiss RA, and Nahorski SR (2003) Non-visual GRKs: are we seeing the whole picture? *Trends Pharmacol Sci* **24**:626–633.

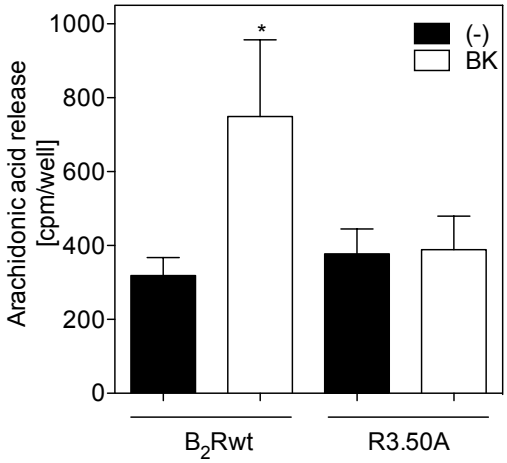
Address correspondence to: Dr. Alexander Faussner, Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Ludwig-Maximilians-Universitaet, Pettenkoferstrasse 9, D-80336 Muenchen, Germany. E-mail: alexander.faussner@med.uni-muenchen.de

Interruption of the ionic lock in the bradykinin B_2 receptor results in constitutive internalization and turns several antagonists into strong agonists

Jasmin Leschner, Goeran Wennerberg, Jens Feierler, Marcel Bermudez, Benjamin Welte, Irina Kalatskaya, Gerhard Wolber, and Alexander Faussner

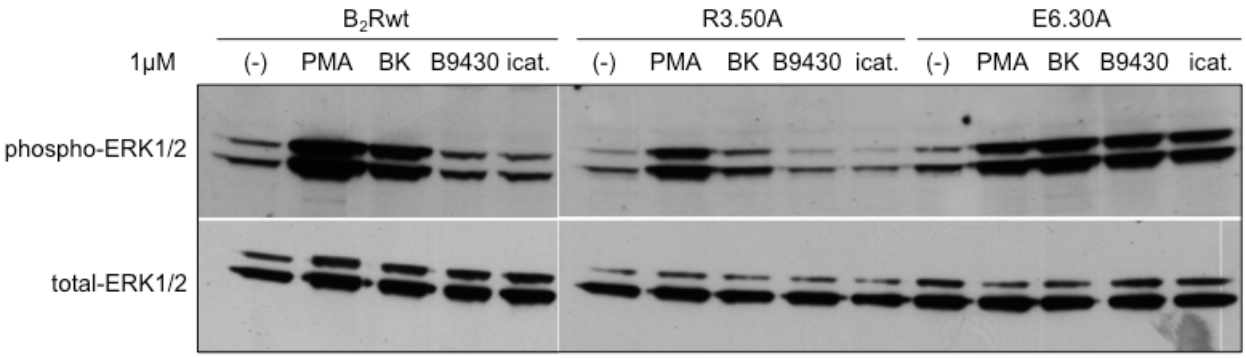


Supplemental Figure 1. Dose-dependent IP accumulation of E6.30A mutant expressing cells in response to B_2R agonist and antagonists. HEK293 cells stably expressing mutant E6.30A and pre-incubated overnight with [^{3}H]inositol, were stimulated with the indicated concentrations of BK, icatibant or JSM10292 for 30 min at 37°C as described in "Material and Methods". Shown is a representative experiment that was repeated three times with similar results.

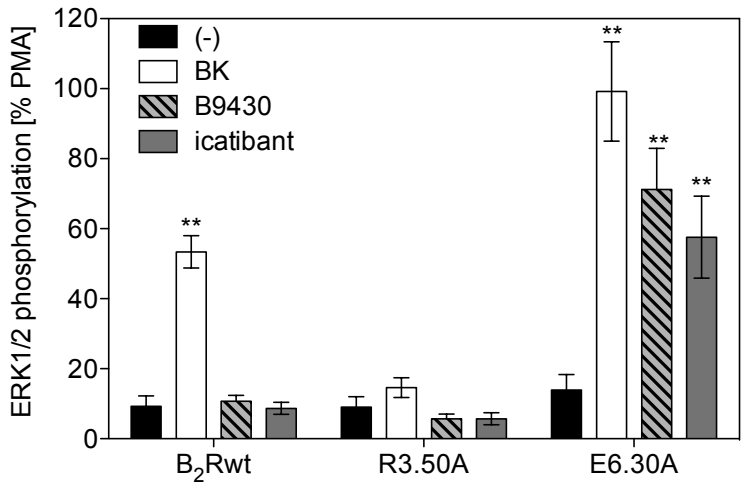


Supplemental Figure 2. Arachidonic acid release. HEK293 cells stably expressing B_2Rwt and R3.50A were cultured in 24-wells. To each well 0.5 μCi [^{3}H]arachidonic acid was added and cultivation was continued for 18 h. Thereafter, cells were rinsed with 37°C warm medium containing 2 mg/ml fatty acid free BSA (bovine serum albumin) and further incubated for 1 h at 37°C in the same medium. Subsequently, cells were washed once with BSA-containing medium and incubated in 300 μl of the same medium in the absence or presence of 1 μM BK for 20 min at 37°C. The release of [^{3}H]arachidonic acid and its metabolites was determined by measuring 200 μl of the cell supernatant in a β-counter after addition of scintillation liquid. Data represent means ± SEM of three independent experiments performed in triplicate. (Comparison of non-stimulated (-) and BK-stimulated arachidonic acid release: $^*P < 0.05$).

A



B



Supplemental Figure 3. B_2R-stimulated activation of ERK1/2. (A) Western blot of B_2R-induced phospho-ERK1/2 and total-ERK1/2. Cells stably expressing B_2Rwt, mutant R3.50A, or mutant E6.30A were serum-starved in Opti-MEM overnight. They were either left unstimulated (-) or treated with 1 µM BK, B9430 or icatibant (icat.) for 10 min at 37°C as indicated. Treatment with 1µM PMA (phorbol-12-myristate-13-acetate, Calbiochem), a potent protein kinase C activator, served as a reference for maximal response (=100%). Protein extraction was performed as described in "Material and Methods (Receptor phosphorylation)" and detection of phospho- and total-ERK1/2 levels (loading control) was carried out using the appropriate antibodies (monoclonal, Cell Signaling, dilution 1/2000). The blot shown is representative of three experiments. **(B) Quantification.** PMA treatment served as positive control and reference for maximal response (=100%). Phospho-ERK1/2 levels were normalized to PMA stimulation and total-ERK1/2 levels in each cell line. Values represent means ± SEM of three independent experiments. (One-way ANOVA with Dunnett's Multiple Comparison Test; Comparison of non-stimulated (-) versus stimulated: $^{**}P < 0.01$).

Danksagung

Besonderer Dank gilt meinem Doktorvater, PD Dr. Alexander Faußner, der mir die Doktorarbeit in seiner Arbeitsgruppe erst ermöglichte. Mit fachlichen Ratschlägen und wissenschaftlichen Anregungen stand er mir stets zur Seite und konnte mich durch seine Begeisterung ermutigen und motivieren. Durch seine angenehm humorvolle und verständnisvolle Art sorgte er immer für ein gutes Arbeitsklima in unserer Gruppe. Für seine fortwährende Unterstützung möchte ich mich recht herzlich bedanken.

Frau Prof. Dr. Marianne Jochum möchte ich herzlich für die Möglichkeit zur Ausübung meiner Tätigkeit als Doktorandin und für die Organisation der Winterschool in Tiers danken.

Insbesondere danke ich meinen ehemaligen Kollegen Dr. Jens Feierler und Dr. Göran Wennerberg, deren Hilfsbereitschaft und offenes Wesen mir den Einstieg in die Laborarbeit erleichterte. Hervorheben möchte ich an dieser Stelle Dr. Jens Feierler, der mir bei Fragen stets engagiert zur Seite stand und immer ein offenes Ohr für mich hatte. Ein besonderes Dankeschön möchte ich auch an Larisa Ring richten, die den Laboralltag durch ihre angenehme Art aufhellte. Ich bin froh in diesen lieben Menschen nicht nur tolle Kollegen, sondern auch sehr gute Freunde gefunden zu haben.
Ich danke auch Cornelia Seidl, Steffen Schüssler und Heidrun Grondinger, deren experimentelle Unterstützung zum Gelingen dieser Dissertation beitrug. Ebenso danke ich Annemarie Oettl, die den Alltag im Labor durch ihr freundliches Wesen bereicherte.

Ich möchte den Institutsmitgliedern außerhalb unserer Arbeitsgruppe danken. Dr. Virginia Egea und Dr. Tanja Popp standen mir stets hilfsbereit und aufmunternd zur Seite. Besonderer Dank gebührt Peter Killian, der mich durch sein Vertrauen stets in meinen Vorhaben bestärkte. Auch bei Maresa Grundhuber, Stefan Simon und Steffen Kraus, die zu der unterhaltsamen und freundschaftlichen Atmosphäre in unserem Institut beitrugen, möchte ich mich von Herzen bedanken.

Ein besonderes Dankeschön gilt Karola Rügamer-Biese und Evelyn Themel, die sich zuverlässig um alle Büro-Angelegenheiten kümmerten.

Für das fröhliche und kollegiale Umfeld danke ich außerdem PD Dr. Dorit Nägler, PD Dr. Christian Ries, PD Dr. Peter Neth und allen hier nicht genannten Institutsmitarbeitern. Für den Beistand in allen Fragen rund um die EDV möchte ich mich ganz herzlich bei Prof. Dr. Christian Sommerhoff bedanken.

Ganz besonderer Dank gilt meiner Familie, die mich stets und in jeglicher Hinsicht unterstützt hat. Meinen Eltern danke ich dafür, dass sie mich zu meiner Doktorarbeit ermutigten und mich lehrten, an neue Herausforderungen mit Mut und Ehrgeiz heranzugehen. Dabei konnte ich jederzeit auf ihren Rückhalt zählen. Ausgesprochen dankbar bin ich auch meiner Schwester, die stets hinter mir stand und auf die ich mich immer verlassen konnte.