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

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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.

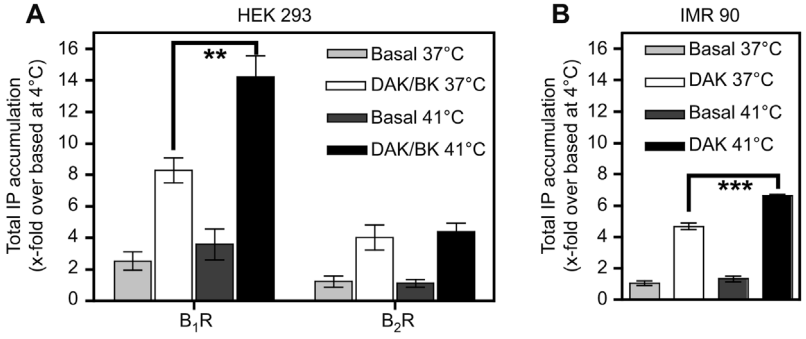

Figure 1 B_1R- and B_2R-stimulated accumulation of inositol phosphates at 37°C vs. 41°C.
(A) HEK 293 cells, stably expressing comparable amounts of either B_1R or B_2R, were stimulated in the presence of 50 mM LiCl for 30 min with 1 μM of the appropriate agonist (DAK or BK) at the indicated temperature. 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.

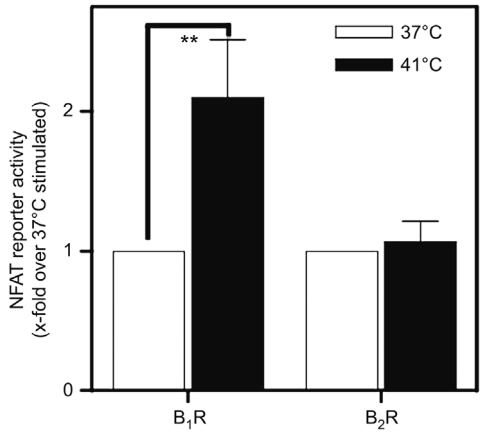

Figure 2 B_1R- and B_2R-mediated induction of NFAT-regulated Gaussia luciferase activity.
NFAT reporter gene host cells on 24-wells transiently expressing B_1R or B_2R were stimulated in DMEM containing 0.5% FCS with 1 μM of the appropriate agonist (DAK or BK) for 10 min at 37°C or 41°C. After further 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-

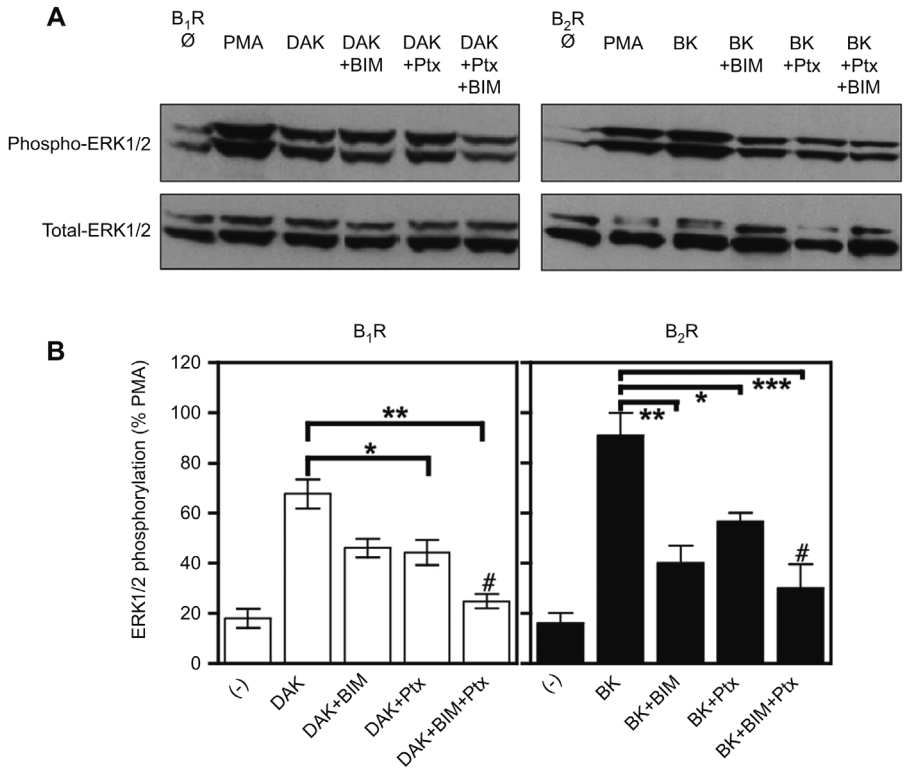

Figure 3 B_1R- and B_2R-stimulated activation of ERK1/2 involving G proteins $G_{q/11}$ and G_i.
(A) Western blot of phospho- and total-ERK1/2. HEK 293 cells stably expressing B_1R and B_2R were serum-starved overnight, pre-treated for 30 min with 5 μM BIM or for 16 h with 75 ng/ml Ptx and stimulated 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

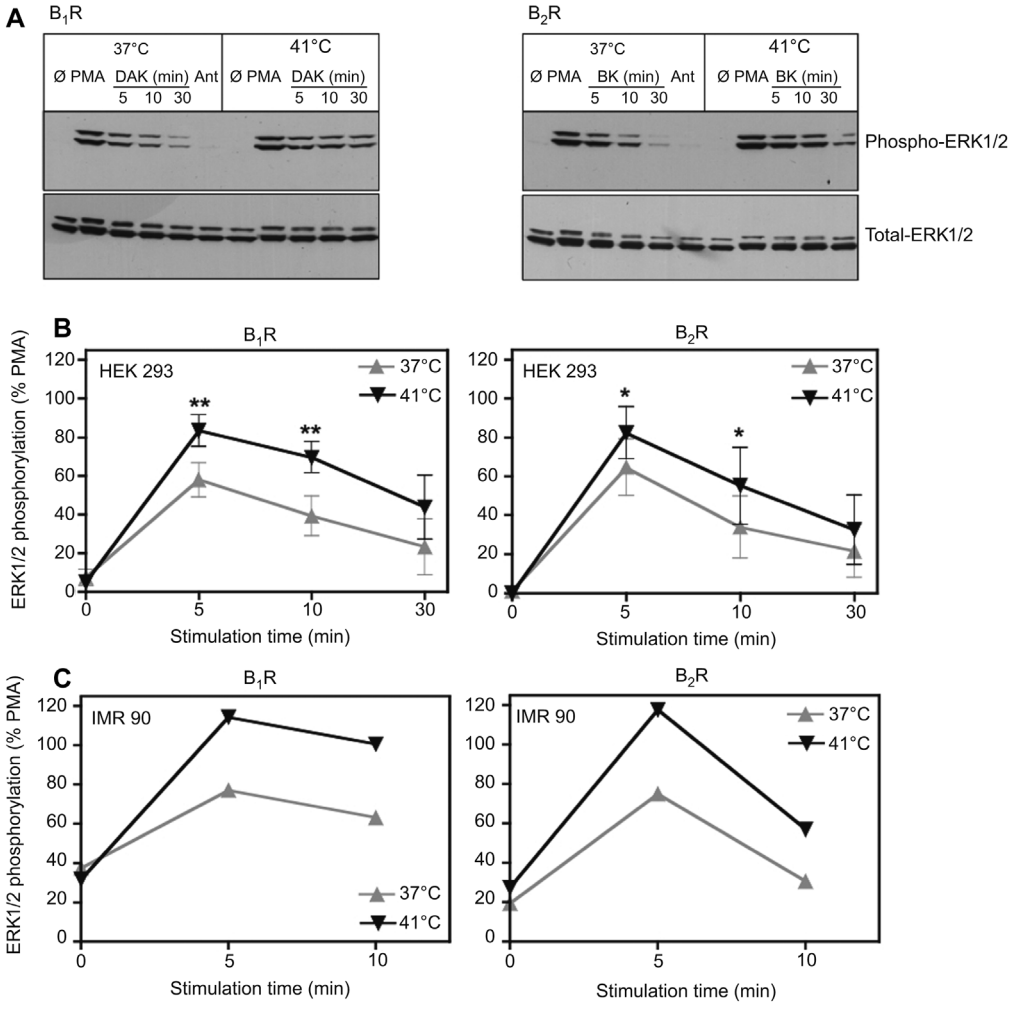

Figure 4 B_1R- and B_2R-induced ERK1/2 activation.
(A) Western blot of phospho- and total-ERK1/2. HEK 293 cells stably expressing B_1R or B_2R were serum-starved overnight, stimulated with 100 nM DAK/BK with or without pre-incubation with the appropriate antagonist (Ant) at 37°C and 41°C for the indicated 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

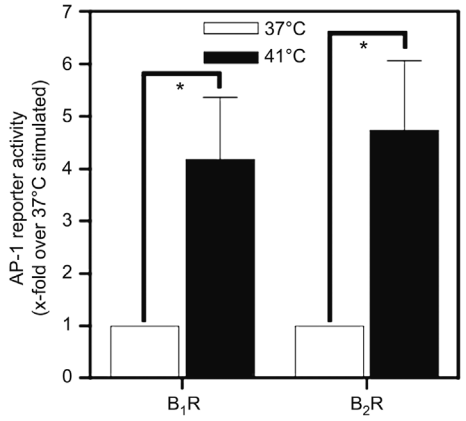

Figure 5 B_1R- and B_2R-mediated induction of AP-1-regulated Gaussia luciferase activity.
AP-1 reporter gene host cells on 24-wells transiently expressing B_1R or B_2R were stimulated in DMEM containing 0.5% FCS with 1 μM of the appropriate agonist (DAK or BK) for 10 min at 37°C or 41°C. After further 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: $^*p<0.05$.)

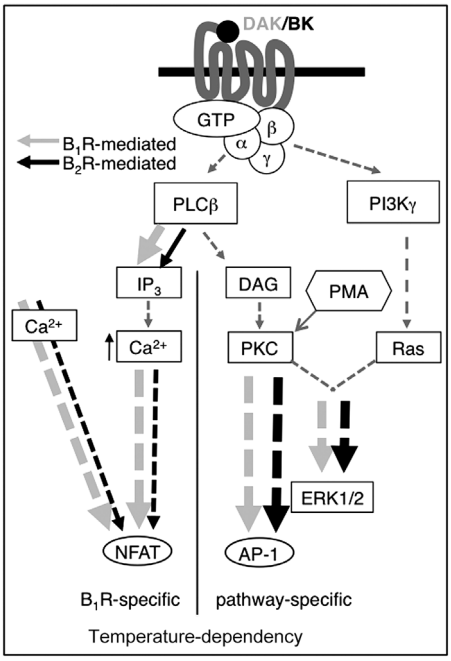

Figure 6 Overview of B_1R- and B_2R-mediated pathways at 37°C vs. 41°C.
Bold arrows indicate increased signal activity at 41°C as compared to 37°C. Light gray arrows show B_1R-specific temperature dependence, while black arrows show pathway-specific temperature dependency. Both kinin receptors are temperature-sensitive.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

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