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Calreticulin enhances B2 bradykinin receptor maturation and heterodimerization

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ABSTRACT

In different native tissues and cells the receptor for the vasodepressor bradykinin, B_2, forms dimers with the receptor for the vasopressor angiotensin II, AT_1. Because AT_1/B_2 heterodimers may contribute to enhanced angiotensin II-stimulated signaling under pathophysiological conditions, we analyzed mechanisms of AT_1/B_2 heterodimerization. We found that efficient B_2 receptor maturation was a prerequisite for heterodimerization because only the fully mature B_2 receptor was capable to interact with AT_1. To identify chaperones involved in B_2 receptor maturation and heterodimerization we performed microarray gene expression profiling of human embryonic kidney (HEK293) cells. The expression of the chaperone calreticulin was up-regulated in cells with efficient B_2 receptor maturation. Vice versa, upon down regulation of calreticulin expression by RNA interference, B_2 receptor maturation and AT_1/B_2 receptor heterodimerization were significantly impaired. Concomitantly, the B_2 receptor-mediated enhancement of AT_1-stimulated signaling was reduced. Thus, calreticulin enhances B_2 receptor maturation and heterodimerization with AT_1.

Introduction

Angiotensin II is an important peptide hormone regulating vascular tone and blood pressure. The vasopressor actions of angiotensin II are mediated mainly by the AT_1 receptor. In contrast, bradykinin is a vasodepressor peptide, which exerts major effects via the B_2 bradykinin receptor. The angiotensin II and bradykinin systems are intertwined at multiple levels. The angiotensin-converting enzyme (ACE) regulates the availability of both peptides in vivo by releasing angiotensin II and inactivating bradykinin. With more than 30% identity, the AT_1 receptor is one of the closest relatives of the B_2 receptor. In agreement with the high homology, a direct interaction between the angiotensin II AT_1 receptor and the bradykinin B_2 receptor in vivo in rat brain is known since 1993 [1]. We followed those original studies, and identified AT_1/B_2 receptor heterodimers in different other native systems and in patients with preeclampsia [2,3]. In addition to the in vivo data, AT_1/B_2 interactions were also reconstituted in a transfected in vitro system [2,3].

Heterodimerization of AT_1 with B_2 receptors leads to signal enhancement of AT_1 [2,3]. Signal enhancement as a consequence of heterodimerization with the B_2 receptor is also effective for the closely related angiotensin II AT_2 receptor [4]. Both studies were performed with native cells [3,4]. Thus, a native, correctly folded B_2 receptor protein seems to be required for receptor heterodimerization and signal enhancement.

For various G-protein-coupled receptors, conditions of efficient protein folding were investigated. Different chaperones are known to be involved in the correct folding of AT_1 receptors such as BiP, calnexin, and D_1RiP78 [5]. By contrast, little is known about chaperones, which assist the folding of B_2 receptors and AT_1/B_2 receptor heterodimerization. We therefore analyzed requirements for efficient B_2 receptor maturation and the potential interrelationship between B_2 receptor maturation and heterodimerization with the angiotensin II AT_1 receptor.

Materials and methods

Cell culture and cell transfection. HEK293 cells were routinely grown in DMEM (100 mg/dl glucose) supplemented with 10% (v/v) FCS unless otherwise indicated. Cells were transfected with Lipofectamine Plus (Invitrogen). Transfection efficiency of HEK293 cells was $\geqslant$90%. Plasmids encoding the human B_2 and AT_1 receptor under control of the CMV promoter were used. Cellular inositol phosphate levels were determined similarly as described [2]. For down-regulation of calreticulin expression, HEK293 cells were transfected with stealth RNAi (20 pmol/ml) targeting the coding sequence of the human calreticulin cDNA (nucleotides 59–83, RNAi-Calreticulin1 and nucleotides 367–391, RNAi-Calreticulin2). Quantification of B_2

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receptors was performed on HEK cells with 50 nM of [^{125}I]-labeled F(ab)$_2$ fragments of affinity-purified B_2 receptor-specific antibodies (~1 μCi) similarly as described [6].

Protein detection in immunoblot and co-enrichment of receptors. Protein detection in immunoblot was performed with membranes prepared by sucrose density gradient centrifugation followed by partial enrichment as described [2]. For co-enrichment of AT_1 and B_2 receptors, membranes of HEK cells were solubilized with RIPA buffer (including protease inhibitor cocktail) and subjected to immuno-affinity chromatography by anti-AT_1 receptor antibodies using 0.1 ml immunoaffinity matrix (Affigel 10; 15 mg affinity-purified antibodies per ml gel). After extensive washing, proteins were eluted with 0.2 M glycine, pH 2.5, neutralized, desalted, delipidated and precipitated [2]. Eluted proteins were dissolved and separated by urea-containing SDS–PAGE under reducing conditions. Enriched AT_1 and co-enriched B_2 receptors were identified in immunoblot with F(ab)$_2$ fragments of the respective anti-receptor antibodies. Enrichment of the B_2 receptor was performed analogously with anti-B_2 receptor antibodies. Chemical deglycosylation of the enriched B_2 receptor was performed with trifluoromethanesulfonic acid (TFMS) at 0 °C as described [7].

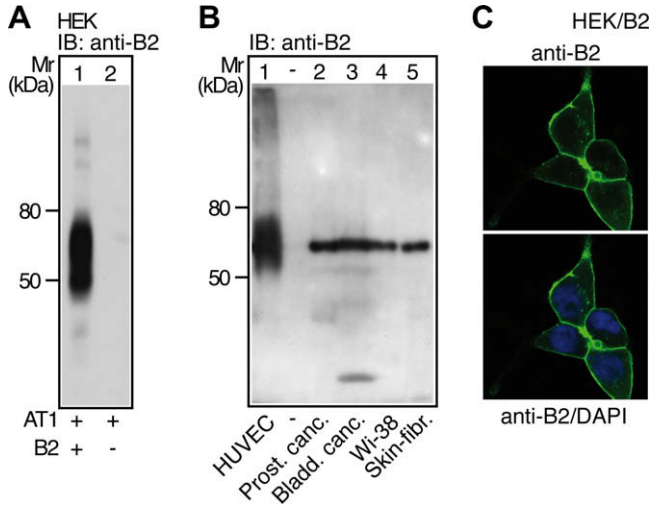

Fig. 1. (A) Immunoblot detection of the B_2 receptor (IB: anti-B2) with F(ab)$_2$ fragments of affinity-purified B_2-specific antibodies on enriched membranes of HEK293 cells (HEK) expressing ~5 pmol B_2 receptor/mg protein (lane 1). As a control, AT_1 receptor-expressing HEK cells were used (lane 2). (B) Immunoblot detection of B_2 receptors on the enriched and acetone-precipitated, solubilized membrane fraction prepared from different native tissues, i.e. human umbilical vein endothelial cells (HUVEC, lane 1), prostate cancer tissue (Prost. canc., lane 2), bladder cancer tissue (Bladd. canc., lane 3), WI-38 fibroblasts (lane 4), and human skin fibroblasts (Skin-fibr., lane 5). (C) Immunofluorescence detection of the B_2 receptor on B_2 receptor-transfected HEK293 cells (HEK/B2). The lower panel shows merged pictures of B_2 receptor localization and DAPI-stained cell nuclei (anti-B2/DAPI). (Original magnification, 630×.)

Antibodies used for immunoblotting and immunofluorescence. All antibodies used for immunoblotting, receptor immunoaffinity enrichment and immunofluorescence were characterized in previous studies [2,3,6,8–10]. Immunofluorescence was performed with paraformaldehyde-fixed (4% w/v) cells [6].

Microarray gene expression profiling. For microarray gene expression analysis, HEK293 cells were cultivated for 7 days in DMEM supplemented with 10% FCS and 100 mg/dl (low) or 450 mg/dl (high) glucose. Total RNA was isolated with the RNeasy Mini kit (Qiagen). Procedures for cDNA synthesis, labeling and hybridization were carried out according to the protocol of the manufacturer (Affymetrix GeneChip Expression Analysis Technical Manual; Rev. 5). For hybridization, 15 μg of fragmented cRNA were incubated with the chip (Affymetrix GeneChip Human genome U133 Plus 2.0 Array) in 200 μl of hybridization solution in a Hybridization Oven 640 (Affymetrix) at 45 °C for 16 h. GeneChips were then washed and stained using the Affymetrix Fluidics Station 450. Microarrays were scanned with the Affymetrix GeneChip Scanner 7G, and the signals were processed using GCOS (v. 1.4, Affymetrix). Gene expression data are available at GEO Accession No. GSE15575.

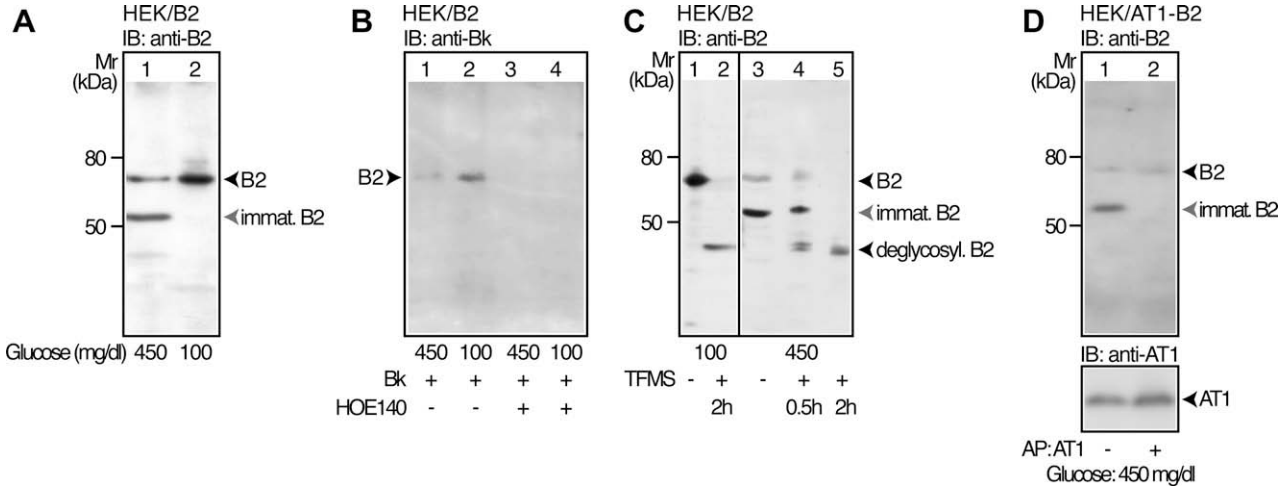

Fig. 2. (A) Immunoblot detection of the B_2 receptor (IB: anti-B2) on enriched membranes of HEK293 cells (HEK/B2; expression of ~240 fmol B_2/mg protein) cultivated in a medium with high (450 mg/dl) or low (100 mg/dl) glucose. (B) Affinity-crosslinking of bradykinin (Bk) to the B_2 receptor expressed in HEK293 cells (HEK/B2) cultivated in medium with 450 or 100 mg/dl glucose as indicated (450; 100), and detection of cross-linked bradykinin in immunoblot with affinity-purified anti-bradykinin antibodies (IB: anti-Bk). B_2-specificity was assessed with the B_2-specific antagonist HOE140 (2 μM; lanes 3 and 4). (C) Chemical deglycosylation (deglycosyl.) by TFMS (±TFMS) of enriched B_2 receptors from HEK293 cells (HEK/B2) cultivated in medium containing 100 or 450 mg/dl glucose followed by immunoblot detection of B_2 (IB: anti-B2). (D) Immunoaffinity enrichment of the AT_1 receptor (±AP: AT1) from AT_1 and B_2 receptor co-expressing HEK293 cells (HEK/AT1-B2) cultivated in medium containing high glucose (450 mg/dl), and detection of co-enriched B_2 receptor or enriched AT_1 receptor in immunoblot with F(ab)$_2$ fragments of affinity-purified B_2- or AT_1-specific antibodies, respectively (lane 2, upper and lower panel). As a control, the solubilisate was applied for immunoblot detection of the B_2 or AT_1 receptor (lane 1, upper and lower panel).

Statistics. Unless otherwise stated, data are expressed as mean ± SE. To determine significance between two groups, we made comparisons using the unpaired two-tailed Student's *t*-test. *p* values of <0.05 were considered significant.

Results

Transfected HEK293 cells synthesize a monomeric B_2 receptor that resembles the native B_2 receptor protein

Protein maturation was assessed with HEK293 cells as an expression system because the B_2 receptor protein of transfected HEK293 cells appeared as a pure monomer (Fig. 1A). Even at high receptor expression levels (>5 pmol/mg protein), the B_2 receptor expressed in HEK293 cells did not show significant protein aggregation under reducing conditions of SDS–PAGE supplemented with urea (Fig. 1A). The B_2 receptor protein of transfected HEK293 cells showed a similar apparent molecular mass in SDS–PAGE as the native B_2 receptor protein of various native tissues and cells (Fig. 1B). Detection of the B_2 receptor by immunofluorescence revealed the predominant membrane localization of the transfected B_2 receptor on HEK293 cells (Fig. 1C). Altogether, these findings indicate that HEK293 cells express all the proteins necessary for efficient B_2 receptor folding.

A

Low glucose increases the general chaperone system of HEK cells

Gene name	Accession no.	Fold increase (low/high gluc.)	Description	No. of increased probe sets (≥2.5-fold)
DNAJB9	gb:NM_012328	6.36	DNAJ (HSP40) homolog, subfamilyB, member 9	1
HYOU1 (ORP150)	gb:NM_006389	5.68	Hypoxia up-regulated 1	1
HSPA5 (GRP78/BIP)	gb:NM_005347	4.97	Heat shock 70kDa protein (glucose-regulated protein, 78kDa)	2
PPP1R15A (GADD34)	gb:NM_014330	4.96	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	2
PDIA4 (ERP70)	gb:NM_004911	3.85	Protein disulfide isomerase family A, member 4	2
DNAJB5	gb:NM_001135004	3.47	DNAJ (HSP40) homolog, subfamilyB, member 5	1
ARMET	gb:NM_006010	3.34	Arginine rich, mutated in early stage tumors	1
SEL1L	gb:NM_005065	3.16	Sel-1 suppressor of lin-12-like (C. elegans)	2
FKBP2	gb:NM_004470	2.79	FK506-binding protein 2 (13 kDa)	1
OS-9	gb:NM_001017956	2.74	Amplified in osteosarcoma	1
SDF2L1	gb:NM_022044	2.68	Stromal cell-derived factor 2-like 1	1
HSEC61	gb:NM_013336	2.63	Homo sapiens sec61 homolog	1
CALR	**gb: NM_004343**	**2.50**	**Calreticulin**	**1**
CANX	gb: NM_001746	2.50	Calnexin	1

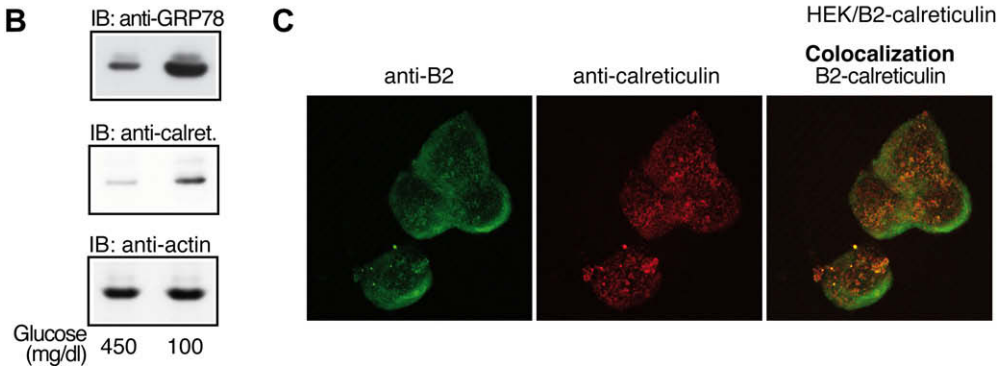

Fig. 3. (A) Microarray gene expression profiling of HEK293 cells (HEK). Probe sets of low glucose-cultivated HEK293 cells with a ⩾2.5-fold increased signal relative to that of high glucose-cultivated HEK293 cells (fold increase; low/high gluc.; *t*-test *p* value ⩽ 0.01), ER localization and involvement in the general chaperone system are listed. (B) Immunoblot analysis controlling the microarray data. (C) Co-localization of the B_2 receptor with calreticulin in HEK293 cells (HEK/B2-calreticulin) cultivated in low glucose medium. (Original magnification: 630×.)

High glucose induces an immature B_2 receptor protein in HEK293 cells

Expression of mature and correctly folded receptors on the plasma membrane depends on the general chaperone system of the endoplasmic reticulum (ER) controlling protein synthesis, folding and assembly [11]. To assess the impact of the general chaperone system on B_2 receptor folding and AT_1/B_2 receptor heterodimerization, we down-regulated the general chaperone system of HEK293 cells by high glucose [12]. Cultivation of HEK293 cells in a standard medium with high glucose (450 mg/dl) led to the appearance of an "immature" B_2 receptor-reactive band of ~53 ± 4 kDa in addition to the (mature) B_2 receptor of 67 ± 5 kDa (Fig. 2A).

The B_2 receptor-reactive band of 53 kDa did not show a high-affinity interaction with the agonist bradykinin as assessed by affinity cross-linking (Fig. 2B). Chemical deglycosylation of the "mature" and "immature" B_2 receptor proteins produced the deglycosylated B_2 receptor of ~43 kDa (Fig. 2C). These experiments strongly suggest that the 53 kDa protein is indeed an immature B_2 receptor which is not fully glycosylated. Interestingly, the immature B_2 receptor of 53 kDa did not interact with the AT_1 receptor as assessed by co-immunoenrichment (Fig. 2D). Thus, efficient B_2 receptor maturation seems to be required for heterodimerization with the AT_1 receptor.

Microarray gene expression profiling of the general chaperone system of HEK293 cells

To determine chaperone(s) involved in B_2 receptor maturation and AT_1/B_2 receptor heterodimerization, we performed microarray gene expression profiling of HEK293 cells cultivated in low and high glucose medium, respectively. Differentially expressed proteins of the ER protein quality-control system were identified by selection of probe sets with (i) ⩾2.5-fold higher expression in low versus high glucose, (ii) ER localization according to gene ontology (GO) analysis, and (iii) involvement in the general chaperone system according to the literature (Fig. 3A). The approach identified 14 ER-localized members of the protein quality-control system that showed a more than 2.5-fold higher expression signal in cells cultivated in low glucose relative to high glucose (Fig. 3A). The microarray gene expression data were verified by immunoblotting as exemplified for GRP78 (Fig. 3B).

The chaperone calreticulin co-localizes with the B_2 receptor

Several of the identified proteins of the general chaperone system are reported to be involved in folding of membrane proteins [5]. Searching for chaperones that are specifically required for the maturation of B_2 receptors, we focused on calreticulin, because gene inactivation studies revealed an interrelationship between calreticulin and functional B_2 receptor protein levels [13]. Immunoblotting confirmed the microarray-based evidence of a significantly higher calreticulin protein expression in HEK293 cells cultivated in low glucose relative to high glucose (Fig. 3B, middle panel). As a control, actin protein levels of HEK293 cells were not significantly different (Fig. 3B, lower panel). In agreement with a potential role of calreticulin in B_2 receptor folding and maturation, immunofluorescence revealed the cellular co-localization of the B_2 receptor with calreticulin (Fig. 3C).

Down-regulation of calreticulin expression by RNA interference impairs B_2 receptor maturation and AT_1/B_2 receptor heterodimerization

To determine the potential involvement of calreticulin in B_2 receptor maturation, we down-regulated the expression of calreticulin by RNA interference. Upon down-regulation of calreticulin, the maturation of the B_2 receptor protein was significantly affected as evidenced by the appearance of the immature B_2 receptor pro-

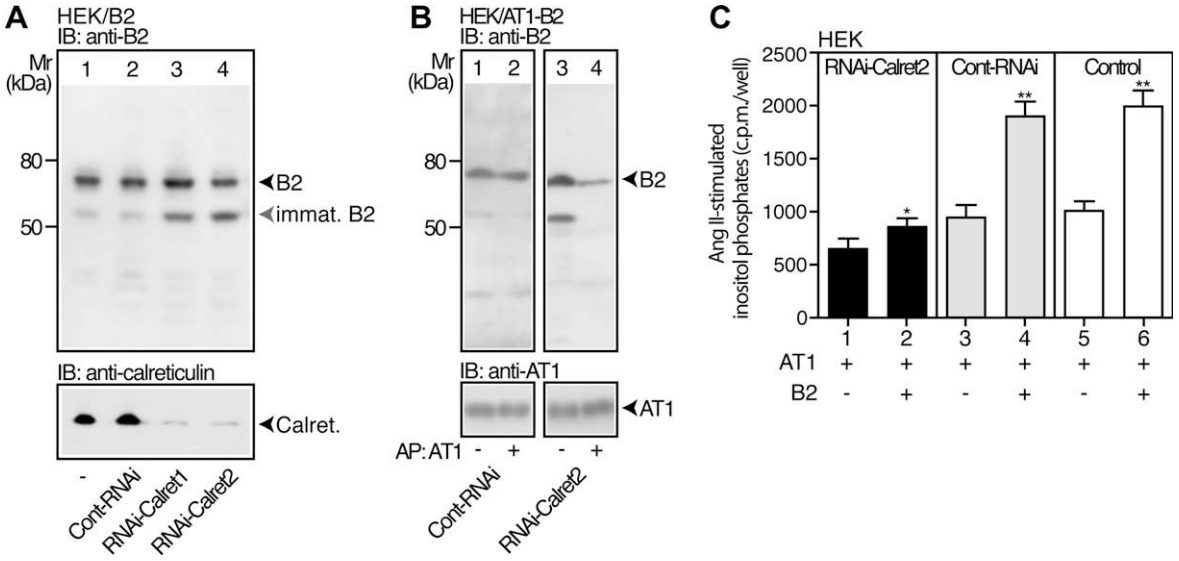

Fig. 4. (A) Immunoblot detection of the B_2 receptor (IB: anti-B2) expressed in HEK293 cells (HEK/B2) cultivated in low glucose (100 mg/dl). As indicated, membranes of control HEK/B2 cells (−; lane 1), of cells transfected with an unrelated control RNAi duplex (Cont-RNAi; lane 2), or of cells transfected with two different RNAi duplexes targeting calreticulin by RNA interference (RNAi-Calret1, RNAi-Calret2; lanes 3 and 4) were applied. The lower panel shows an immunoblot of calreticulin (IB: anti-calreticulin). (B) Immuno-affinity enrichment of the AT_1 receptor (±AP: AT1) from AT_1 and B_2 receptor co-expressing HEK293 cells (HEK/AT1-B2) transfected with an unrelated control RNAi duplex (Cont-RNAi; left panels) or an RNAi duplex targeting calreticulin by RNA interference (RNAi-Calret2; right panels), and detection of the co-enriched B_2 receptor or enriched AT_1 receptor in immunoblot with $F(ab)_2$ fragments of affinity-purified B_2- or AT_1-specific antibodies, respectively (lanes 2/4, upper and lower panels). Lanes 1 and 3 show detection of the B_2 or AT_1 receptor in the solubilisate. (C) Strongly reduced B_2 receptor-mediated enhancement of the angiotensin II-stimulated (AngII, 100 nM) inositol phosphate signal upon down-regulation of calreticulin (columns 1 and 2). As a control, transfection of an unrelated control RNAi duplex (Cont-RNAi) did not impair the B_2 receptor-mediated enhancement of the angiotensin II-stimulated inositol phosphate generation relative to control cells (Control) without RNAi duplex transfection (columns 3 and 4 versus 5 and 6). HEK293 cells expressed comparable levels of cell-surface receptors as determined with [^{125}I]-labeled $F(ab)_2$ fragments of affinity-purified B_2- and AT_1-specific antibodies (268 ± 9 fmol/mg protein of B_2 receptor and 139 ± 8 fmol/mg protein of AT_1 receptor). Data represent mean ± SE, $n = 3$ ($^{*}p < 0.04$; $^{**}p < 0.002$).

tein band of 53 kDa even when cells were cultivated in a medium with a physiological (low) glucose concentration (Fig. 4A).

Moreover, down-regulation of calreticulin strongly reduced the AT_1/B_2 receptor interaction because only the fully mature B_2 receptor of 67 kDa was capable to interact with AT_1 whereas the immature B_2 receptor of 53 kDa was not significantly co-enriched with AT_1 (Fig. 4B). Concomitantly, the B_2 receptor-mediated enhancement of the AT_1-stimulated signal—an additional indicator of AT_1/B_2 receptor heterodimerization—was strongly decreased upon down-regulation of calreticulin (Fig. 4C). Together these data suggest that endogenous calreticulin expression levels of HEK293 cells are sufficient to enhance B_2 receptor maturation and AT_1/B_2 receptor heterodimerization.

Discussion

In various in vivo systems the receptor for the vasodepressor bradykinin, B_2, undergoes a functional interaction with the receptor for the vasopressor angiotensin II, AT_1 [1–3,14]. The covalently bonded AT_1/B_2 receptor heterodimers are distinguished from individual, dissociable receptors by a kinetically favored interaction with G-proteins leading to enhanced AT_1-stimulated signaling [2–3]. Because increased formation of AT_1/B_2 receptor heterodimers could constitute a pathological feature of hypertensive disorders [3,14], the current study investigated mechanisms required for functional AT_1/B_2 receptor heterodimerization.

Several lines of evidence are presented supporting that efficient protein maturation of the B_2 receptor is a prerequisite for functional interaction with AT_1. First, only the mature B_2 receptor interacted with co-expressed AT_1 as determined by co-enrichment. Second, microarray gene expression profiling revealed a correlation between efficient AT_1/B_2 receptor heterodimerization and the expression of the general chaperone system. Finally, RNA interference studies determined the involvement of the chaperone calreticulin in B_2 receptor maturation and AT_1/B_2 receptor heterodimerization. Future studies will have to assess whether the herein identified requirement of specific chaperone(s) for AT_1/B_2 receptor heterodimerization is a factor involved in the induction of AT_1/B_2 receptor heterodimers under pathophysiological conditions.

Acknowledgment

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