Regulation of Cardiovascular Signaling by Kinins and Products of Similar Converting Enzyme System

Expression levels strongly affect ligand-induced sequestration of B₂ bradykinin receptors in transfected cells

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Faussner, Alexander, Alexandra Bauer, Irina Kalatskaya, Marianne Jochum, and Hans Fritz. Expression levels strongly affect ligand-induced sequestration of B₂ bradykinin receptors in transfected cells Am J Physiol Heart Circ Physiol 284: H1892–H1898, 2003; 10.1152/ajpheart.01147.2002.—Transfection of cells with expression vectors is one of the most important tools used to assess the effects of receptor mutations on ligand-induced receptor sequestration. Most transfection methods give rise to transiently or stably transfected clones with a wide range of receptor expression levels that may also depend on the mutations made. It is, therefore, important to determine how the regulation of the receptors depends on their numbers per cell. In Chinese hamster ovary (CHO) and human embryonic kidney (HEK)-293 cells expressing high levels of B₂ kinin receptors, we observed poor sequestration indicated by <20% reduction in cell surface receptor number after 10 min of stimulation with 1 μM bradykinin (BK) compared with >70% in low-expressing cells. Whereas the rate of [³H]BK internalization (internalized [³H]BK in percentage of total bound [³H]BK) in low-expressing cells was independent of the ligand-concentration used, in high-expressing cells a strong rate decrease was observed with higher (>1 nM) concentrations. Lower ligand concentrations, however, led to internalization rates identical to those obtained in low-expressing cells. Transiently transfected HEK and COS-7 cells showed results similar to those of stably high-expressing cells. Our results demonstrate the difficulty in determining the internalization pattern of (mutated) B₂ kinin receptors, and possibly of G protein-coupled receptors in general, using a sequestration assay in high-expressing cells or transiently transfected cells with high numbers of receptors per transfected cell. However, the receptor (mutant)-specific internalization rate can be measured, provided that the ligand concentrations used are below a threshold at which the internalization rate is still independent of the ligand concentration.

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MATERIALS AND METHODS

Materials. CHO cells DUXB1 were purchased from American Type Culture Collection, and Flp-In T-REx [human embryonic kidney (HEK)-293] cells were from Invitrogen (Groningen, The Netherlands). [2,3-Prolyl-3,4-³H]brady-

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kinin (108 Ci/mmol) was from Perkin Elmer Life Sciences (Boston, MA). Peptides were bought from Bachem (Heidelberg, Germany). The primers were synthesized by Invitrogen and delivered desalted and lyophilized. **Plaque-forming unit** DNA polymerase was obtained from Stratagene Europe. Roche (Mannheim, Germany) delivered Fugene and Invitrogen Hygromycin B and Blasticidin. Polylysine, captopril, 1.10 phenanthroline, and bacitracin were purchased from Aldrich (Taufkirchen, Germany). Fetal calf serum, culture media, and penicillin-streptomyycin were delivered by PAA Laboratories (Cölbe, Germany). All other reagents were of analytic grade and are commercially available.

**Expression vectors.** The sequence of the human B2 kinin receptor, starting with the third encoded methionine, was cloned into the BamHI and the XhoI sites of the pcDNA5/FRT vector (where FRT represent Flp recombinant target) from Invitrogen. The receptor sequence was preceded at the NH2 terminus by either a single hemagglutinin (MGYP-PYDVPDYAGSA) or a double-tag sequence (MGRSHHH-HHH-GYPYDVPDYAGSA) cloned into the HindIII and BamHI site of the vector. To obtain lower expression levels of the B2 kinase receptor (B2KR) in HEK-293, the cytosyngalovirus (CMV) promoter sequence was remeans from the pcDNA5/FRT by cleavage with MfeI and HindIII and replaced by the simian virus 40 (SV40) promoter sequence derived from the pFRT/lacZeo vector (Invitrogen) using standard PCR technology. Alternatively, the tagged B2KR coding sequences were cloned into a modified pRESneo vector from Clontech. For B2eGFP chimera, the sequence for enhanced green fluorescent protein (eGFP) taken from the pEGFP-C1 vector (Clontech) was added to the COOH terminus of the B2KR with standard PCR methodology using a chimeric primer that excluded the stop codon of the B2KR but included the first methionine of the eGFP-construct. For transient expression in COS-7 cells, the pcDNA3.1 (Invitrogen) was used because the other vectors do not contain the SV40 origin needed for plasmid amplification.

**Cell culture.** CHO cells were cultivated in MEM-α with ribonucleosides and deoxyribonucleosides supplemented with 10% fetal calf serum and penicillin-streptomycin. A CHO host cell line harboring the FRT site was created with the pFRT/lacZeo vector (Invitrogen) according to the instructions of the supplier. HEK-293 cells were grown in DMEM with 10% fetal calf serum and penicillin-streptomycin. Transfection of these cells was done using Fugene (Roche) by following the instructions of the producer. CHO cells on 12-well plates were rinsed three times with PBS, preincubated with incubation buffer (15–45 min), and incubated with 0.3 ml of [3H]BK on ice for 60 min to reach equilibrium binding. To start [3H]BK internalization, the plates were placed in a water bath at 37°C. The internalization process was stopped after the indicated time by putting the plates on ice and washing the monolayers four times with ice-cold PBS. Surface-bound [3H]BK was then separated by treating the cell monolayer with 0.2 ml of an ice-cold dissociation solution (0.2 M acetic acid, 0.5 M NaCl, pH 2.7) for 10 min on ice. The supernatant with formerly surface-bound [3H]BK was quantitatively transferred to a scintillation vial by rinsing the cells with another 0.2 ml of PBS (surface-bound [3H]BK). The remaining cell monolayer containing the internalized [3H]BK was lysed in 0.2 ml 0.3 M NaOH and transferred with another 0.2 ml of water to a scintillation vial. The radioactivity of both samples was determined in a betacounter after 2 ml scintillation fluid were added. Nonreceptor-mediated [3H]BK internalization and surface binding were determined in the presence of 5 μM unlabeled BK and subtracted from total binding to give specific values.

**Protein determination.** Total protein was quantified with the Micro BCA Protein assay reagent kit from Pierce (Rockford, IL) using bovine serum albumin as the standard.

**RESULTS**

**Sequestration of stably expressed B2KR s in HEK-293 and CHO cells.** Using the Flp-In system (Invitrogen), we obtained clones of CHO and HEK-293 cells that stably expressed the human wild-type B2KRs or eGFP chimera thereof in high numbers exhibiting a Bmax of ~4 pmol/mg protein for B2KRs and B2eGFP in CHO cells and 11 pmol/mg protein in HEK-293 cells and values of dissociation constant (Kd) of 1.2–2.9 nM for both cell types and both constructs. Surprisingly, however, the B2KRs and the eGFP chimera of both the HEK-293 and the CHO cells responded to stimulation with 1 μM BK with only very poor sequestration. These data obviously deviated from those we had published earlier with respect to CHO cells (1) (Fig. 1).
Internalization of $[^3H]BK$ by stably and highly overexpressed $B_2$KRs. Internalization of $[^3H]BK$ in these overexpressing HEK-293 cells (expressed as internalized radioactivity in percentage of specifically total bound radioactivity) is strongly dependent on the concentration of ligand applied. Fast internalization occurred with low amounts of ligand ($<1 \text{nM}$), which slowed down drastically with higher concentrations of $[^3H]BK$, from more than 80% internalization at $0.4 \text{nM}$ $[^3H]BK$ after 5 min to as low as 10% at $10 \text{nM}$ $[^3H]BK$ (Fig. 2A).

The apparent decrease of the internalization rate was due in part to a much less pronounced decrease of surface binding at higher concentrations. Whereas at a low concentration of $[^3H]BK$ ($0.4 \text{nM}$) almost all surface-bound ligand was internalized within 5 min, at $8 \text{nM}$ of $[^3H]BK$, there was still a considerable amount of $[^3H]BK$ bound to the surface even after 30 min (Fig. 2B). As indicated in Fig. 2C, the uptake rate by which the cells internalize the ligand is obviously limited, because almost no difference exists in the amount of internalized $[^3H]BK$ within the first 10 min when either 2 or $10 \text{nM}$ of $[^3H]BK$ were applied, despite the fact that at equilibrium on ice, there was approximately three times more ligand bound to the surface at the higher concentration (Fig. 2, B and C). Figure 3 shows a similar concentration dependence of the internalization both for highly expressed $B_2$KRs wild types and $B_2$eGFP-chimera in HEK-293 cells as well as in CHO cells.

$[^3H]BK$ internalization in low-expressing CHO and HEK-293 cells. To test the hypothesis that overexpression of the kinin receptors is responsible for the poor receptor sequestration and the concentration dependence of ligand internalization, we tried to generate clones with lower receptor expression. For this purpose, the pIREs expression system (Clontech) was employed. Usage of this expression vector, which inserts itself rather randomly into the cell genome, resulted in several stable CHO clones with receptor expression levels below $1 \text{pmol/mg}$ protein. Internalization of $B_2$KRs in these low-expressing CHO cells no longer showed a dependence on the concentration of $[^3H]BK$. 

Fig. 1. $B_2$ kinin receptor ($B_2$KR) sequestration in high-expressing cells. Human embryonic kidney (HEK)-293 and Chinese hamster ovary (CHO) cells, stably expressing high numbers of $B_2$KRs, were preincubated with $1 \mu\text{M}$ bradykinin (BK) for the indicated times at $37^\circ\text{C}$. Remaining surface binding was then determined at $0^\circ\text{C}$ with $\sim 2 \text{nM}$ $[^3H]BK$ as described in MATERIALS AND METHODS. The results are given as means $\pm$ SD of triplicate determinations. Curves shown are representative of two [CHO; $B_2$KR and $B_2$ enhanced green fluorescent protein ($B_2$eGFP)] and three [HEK-293; $B_2$KR (twice) and $B_2$eGFP] other experiments with similar results. CHO published curve obtained with a $B_2$KR construct is taken from Faussner et al. (1).
In contrast, we were not able to produce HEK-293 cells with low B2KR expression using the pIRES system. Therefore, we exchanged the strong CMV-promotor regulating the expression of the gene of interest in the pcDNA5/FRT vector of the Flp-In system (Invitrogen) via PCR for the distinctly weaker SV40 promotor (see MATERIALS AND METHODS). With this vector applied, the Flp-In system enabled the production of identical clones with lower expression (Bmax 4.2 pmol/mg protein, KD/H11005 2.26 nM). Internalization of [3H]BK was totally independent of the ligand concentration (Fig. 4B) in these new HEK-293 clones.

B2KR sequestration in low-expressing CHO and HEK-293 cells. Both low-expressing CHO and HEK-293 cells responded to stimulation with 1 uM BK with rapid reduction of the receptor number on the cell surface, reaching a plateau of remaining surface binding of under 30% within 10 min, similar to the results published by us previously (Fig. 5).

Internalization of transiently expressed B2 kinin receptors. Transient transfection of cells with receptor genes is a very common approach to studying receptor-associated mechanisms because results can be obtained rather quickly. However, this method usually does not allow to discriminate between causes of the radioactive binding activity: whether this activity derives from a few cells expressing high amounts or from many cells expressing minor amounts of receptors. In [3H]BK-internalization studies with transiently B2KR-transfected HEK-293 cells (Fig. 6A) and COS-7 (Fig. 6B), cells we observed a clear dependence of the internalization rate on the ligand concentration despite the fact that Bmax with 1 pmol/mg protein (HEK-293) or 1.9 pmol/mg (COS-7) was apparently in the range of our low-expressing cells. This dependence indicates that most of the transiently transfected cells highly overexpressed the receptor. Yet, applying lower amounts of [3H]BK (0.6 nM) in both cell lines gave rise to internalization curves similar to those seen for endogenously expressing cells and for stable clones expressing lower amounts of B2KRs.

DISCUSSION

Transfection of various mammalian cell lines such as CHO, HEK-293, or COS cells, either transiently or stably with receptor genes and their variants, is a powerful and therefore widely applied tool for studying the function of the different domains of GPCRs. The same holds true for the (altered) behavior of these receptors when they are coexpressed with other proteins. The effects that mutations, deletions, and truncations of these receptors exert on their regulation often provide deeper insights into regulation mechanisms involving these receptors.
Stable and transient transfection often results in receptor numbers per cell that are far above those observed in endogenously expressing cells. The advantage of such an overexpression is the relatively low background. However, it has to be made certain that the overexpressed receptors do not differ too much in their behavior from naturally expressed ones. For this reason, experimental conditions have to be defined that enable the correlation of results obtained from artificially expressed wild-type receptors with those from naturally expressed ones, so that the probable different behavior of mutated receptors may be interpreted properly.

Using the Flp-In system, we obtained several clones expressing high amounts of B2KR: 10–11 pmol/mg protein in HEK-293 cells and 4.5 pmol/mg protein in CHO cells. Similar high-expression levels in CHO cells have been reported by others (15). Our results clearly demonstrate that such an overexpression prevents the internalization machinery from rapidly translocating all occupied receptors. This can be deduced either from the poor sequestration of B2KRs after stimulation with saturating amounts of unlabeled agonist or from the slow ligand internalization when concentrations are used that lead to occupation of a major part of the receptor compartment. This happens mostly in internalization assays in which (almost) saturating concentrations of tritiated ligands are used as opposed to assays in which iodinated ligands are used. In the latter, the ligand concentrations are usually far below the $K_d$ due to the high specific labeling.

The data presented indicate that the term “overexpression” has to be interpreted in the context of the cell type used. Whereas a B2KR expression level of 4.2 pmol/mg protein still enabled fast internalization rates in HEK-293 cells (even with almost saturating concentrations of [3H]BK (Fig. 4)), a similar expression level of 4.5 pmol/mg protein in CHO cells led to a clear dependence of the internalization rate on the ligand concentration applied (Fig. 3). This difference may be caused by cell-type specific expression levels of proteins involved in receptor sequestration, as indicated by the different expression levels observed for β-arrestin in CHO, COS-7, and HEK-293 cells (9). Although β-arrestins are involved in the sequestration of many GPCRs via clathrin-coated pits (12), it is possible that they do not play a role in the sequestration of the human B2KR, because coexpression of the B2KR with a dominant-negative β-arrestin mutant had no influence on its sequestration (8). Although phosphorylation of the B2KR is required for its sequestration (14), it is rather unlikely that the phosphorylation by a specific receptor kinase is the rate-limiting step in the sequestration of overexpressed B2KRs because this is a very rapid reaction in which one kinase molecule can phosphorylate many receptors in a very short time. Because there are several reports indicating that the B2KR is sequestering via caveolae (3, 10), it is more probable that the longer-lasting interactions with adaptor pro-

Fig. 6. Internalization in transiently transfected cells. HEK-293 cells (A) and COS-7 cells (B) were transfected transiently by electroporation as described in MATERIALS AND METHODS. After 2 days, the cells expressing ~1 pmol/mg protein (HEK) and 1.9 pmol/mg protein (COS-7) were treated, and the internalization was determined as described in Fig. 2. Each time point represents the means ± SD of triplicate determinations. One other experiment with HEK cells was performed giving similar results.
teins, either those involved in the translocation of the B2KR into the caveolae or those in the following sequestration into intracellular compartments is the rate-limiting step. So far, however, little is known about the exact mechanism of GPCR sequestration via caveolae and therefore little about the kinds of protein-protein interactions that may be responsible for this rate limitation.

Our results indicate that published data on reduced internalization rates of receptor mutants have to be carefully interpreted in particular when these data were obtained from transiently transfected cells, COS-7 cells in particular, or from stable clones with very high expression. For example, it was reported that at an expression level of 8.59 pmol/mg protein in COS-7 the B2 receptor double mutant C324A/C329A reveals a 30% reduction in internalization compared with the wild type (13). Yet, we found a perfectly normal internalization behavior for this mutant in HEK-293 cells with lower expression (not shown).

Cautious interpretation is also necessary when one wants to draw conclusions concerning the recycling of the B2KRs from differences between the receptor sequestration and ligand internalization rates (8). Because determination of receptor sequestration, i.e., the change in the number of receptors on the cell surface after exposure to an agonist, always involves the full receptor complement, it has to be compared with ligand internalization rates under identical conditions, i.e., with (almost) saturating ligand concentrations. Our results show that under these conditions the relative reduction of receptors on the cell surface of high- and low-expressing cells (Figs. 1 and 5) corresponds well with the relative amount of ligand internalized (Figs. 2A and 4, respectively), indicating that there is no significant receptor recycling in the first 30 min after receptor activation.

Whereas the present report deals only with the loss of receptor sequestration in cells that overexpress B2KRs, this loss might be a phenomenon that occurs in other GPCRs systems, too. For example, the vasopressin receptor V1a responds to 20-min exposure to an agonist with an 80% reduction of the number of receptors on the cell surface, when stably expressed in HEK-293 cells (5). However, only a 30% reduction under otherwise identical experimental conditions was observed when V1a is transiently expressed (4).

Another interesting aspect of the phenomenon described here concerns the possibility that these stably overexpressing cells may represent partially sequestration-deficient cell lines. Therefore, one could use these cell lines to identify proteins involved in receptor internalization by transiently overexpressing these proteins in these cells and trying to find out whether they are capable of rescuing receptor-sequestration.

In summary, our results demonstrate that highly expressed B2KRs in HEK and CHO cells no longer show receptor-specific sequestration and internalization rates. To overcome this problem, usage of weaker or inducible expression promoters to obtain lower expression levels might be useful. Alternatively, a internalization assay could be used with a ligand concentration that enables the internalization rate independent of the ligand concentration; meaning that only as many receptors are occupied and activated as the cell can normally deal with. Because receptor wild types and mutants often have quite different expression levels, the highest ligand concentration that still gives rise to specific internalization rates might be considerably different for each, and therefore each has to be checked separately, particularly when a reduction in the internalization rate becomes obvious compared with the wild type.

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