Spontaneous Human B2 Bradykinin Receptor Activity Determines the Action of Partial Agonists as Agonists or Inverse Agonists

EFFECT OF BASAL DESENSITIZATION*

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In this report, we show that desensitization regulates ligand-independent, spontaneous activity of the human B2 bradykinin (BK) receptor, and the level of spontaneous receptor activity determines the action of the BK antagonists and partial receptor agonists NPC17731 and HOE140 as agonists or inverse agonists. Spontaneous receptor activity was monitored by measuring basal cellular phosphoinositide (PI) hydrolysis as a function of the density of the receptor in transiently transfected HEK293 cells. Minimal spontaneous activity of the wild-type B2 receptor was detected in these cells. Mutating a cluster of serines and threonines within the fourth intracellular domain of the receptor, which is critical for agonist-promoted desensitization, significantly increased the spontaneous receptor activity. BK, the natural B2 receptor ligand and, consequently, a full agonist, stimulated PI hydrolysis at high and low levels of spontaneous receptor activity. On the other hand, the partial agonists NPC17731 and HOE140 were stimulatory, or agonists, at the lower level of receptor activity but inhibitory, or inverse agonists, at the higher level of activity. These results show that receptors are desensitized in response to their spontaneous activity. Furthermore, these results, which refute traditional theories, show that the capacity of a drug to modulate a receptor response is not intrinsic to the drug but is also dependent on the cellular environment in which the drug acts.

GPCR1 constitute the largest family of receptors and mediate responses to numerous agonists including hormones, neurotransmitters, and sensory stimuli. Receptor activation by the agonist ligand is considered the first step in receptor signal transduction. However, the recent discovery that GPCR exhibit ligand-independent, spontaneous, and mutation-induced activity has introduced a new function that needs to be considered in cellular signaling and ligand action (1) and that can lead to disease (2–4).

GPCR have evolved as the most common targets for therapeutic drugs (5). Drug efficacy is defined as the capacity of a drug to activate or inactivate a receptor response and is fundamental to pharmacology and drug development. Classically, this parameter has been considered an intrinsic property of the drug and independent of the cellular environment in which the drug acts (6–8). Partial agonists, or mixed agonist/antagonists, are prevalent among GPCR drugs and are defined as ligands that elicit a submaximal receptor response and block the response to the natural receptor ligand, a full agonist, or any other ligand of higher efficacy. It has been predicted that in the absence of a more efficacious agonist, the efficacy and behavior of a partial agonist should be directly dependent upon the level of spontaneous receptor activity (9). In other words, if the partial agonist ligand elicits a response that is higher than the spontaneous activity, then the ligand should behave as an agonist, whereas if the ligand elicits a lower response, then the ligand should block the spontaneous activity and, consequently, behave as an inverse agonist.

Desensitization is classically defined as the fading of a receptor response to persistent agonist stimuli and manifests itself in numerous biological processes. Agonist-promoted desensitization of GPCR is common (10), and the mechanism for this process has been described in considerable detail (11, 12). Desensitization in response to spontaneous receptor activity, or basal desensitization, should also occur but has not been investigated to any significant extent. However, this process represents one cellular mechanism which may regulate spontaneous receptor activity and, consequently, the action of partial receptor agonists.

In this study, we used the human B2 bradykinin (BK) receptor expressed in HEK293 cells to show that receptors are subject to desensitization in response to their spontaneous activity. Furthermore, the level of basal desensitization and, consequently, spontaneous receptor activity determines the efficacy and behavior of two potent and selective BK antagonists and partial B2 receptor agonists as either agonists or inverse agonists at the receptor.

EXPERIMENTAL PROCEDURES

Materials—[3H]prolyl 3,4-3H[NPC17731 (53.5 Ci/mmol) and myo-[3H]inositol (10–20 Ci/mmol) were obtained from NEN Life Science Products. All other chemicals were obtained as previously described (13).

Mutation and Transfection—Mutations and transfections of the human B2 receptor were done as described previously (13). Mutations were made using a polymerase chain reaction-ligation-polymerase chain reaction protocol, and HEK293 and A10 cells were transfected with varying amounts of receptor DNA (0.01–0.2 μg/106 cells) using the calcium phosphate precipitate method and LipofectAMINE, respectively.

Receptor Expression—Expression of receptor constructs was assayed by radioligand binding on intact cells essentially as described previously (14). In short, cells were incubated in Leibovitz’s L-15 medium, pH 7.4, 0.1% bovine serum albumin, including the protease inhibitors bacitracin (140 μg/ml) and 1,10-phenanthroline (1 mM) and a saturating concentration of [3H]NPC17731 (3–5 nM) at 4 °C for 60–90 min. The
To analyze ligand-independent, spontaneous activity of the human WT B2 receptor, basal cellular PI hydrolysis was assayed as a function of the density of the receptor in transiently transfected HEK293 cells (Fig. 1B). The slope of this function, which may be considered an index of the level of spontaneous receptor activity and which we term index of basal activity, or IB, was very low (0.03), indicating that the WT receptor was virtually inactive in the absence of a ligand in these cells. In the presence of 1 μM BK, the slope, which we term index of maximal agonist-promoted activity, or IA, was 0.86, a 23-fold increase over the ligand-independent B2 receptor activity (Fig. 1C). BK activation of the WT receptor also was observed by monitoring the downstream mobilization of intracellular Ca\(^{2+}\) in transiently transfected A10 cells. As shown in Fig. 1D, 0.1 μM BK elicited a rapid and transient increase in the intracellular free Ca\(^{2+}\) concentration in these cells, which returned to nearly baseline levels by 5 min. The absence of any residual receptor-mediated Ca\(^{2+}\) mobilization and the lack of effect of the BK antagonist HOE140 (1 μM) after approximately 5 min of agonist exposure indicated that the WT receptor was desensitized at this time point as described previously (15).

To determine whether the low level of ligand-independent, spontaneous activity of the WT B2 receptor was due to desensitization in response to spontaneous receptor activity, or basal desensitization, we mutated receptor residues critical for agonist-promoted desensitization. This mechanism involves the GRK-mediated phosphorylation of specific clusters of serines and threonines in intracellular domain three and/or four (IC-IV) of the receptor (11, 12). As shown in Fig. 1A, the human B2 receptor IC-IV contains several serines and threonines, including Ser315, Ser339, Thr342, Thr345, Ser346, and Ser348, which are conserved among known B2 receptors and which may serve as GRK substrates. Furthermore, BK stimulates serine and threonine phosphorylation of the human B2 receptor (16). To screen for the role of these residues in the regulation of ligand-independent receptor activity, alanine mutants of each individual conserved residue were assayed for basal PI hydrolysis at approximately equal receptor densities (~100 fmol/10\(^6\) cells). As shown in Fig. 2 (left panel), individual mutation of Ser315, Thr342, Thr345, and Ser348 yielded small but significant increases (1.4–1.5-fold) in basal PI hydrolysis, whereas mutation of Ser316 and Ser346 had essentially no effect. Interestingly, alanine mutation of Thr342, Thr345, Ser346, and Ser348 (SerThr cluster) as a cluster to yield B2ASerThr resulted in an increase (5.1-fold) in basal PI hydrolysis, which was much higher than that seen with each individual mutation alone. Furthermore, expression of increasing amounts of B2ASerThr yielded an IB of 0.19 which was 6-fold greater than that of the WT B2 receptor which was 0.03 (Fig. 1B). These results show that the human WT B2 receptor is phosphorylated and desensitized in response to spontaneous receptor activity and that this process regulates the spontaneous activity of the receptor. The phosphorylation apparently involves primarily Ser315, Thr342, Thr345, and Ser348 in IC-IV. Considering the effect of mutating these residues as a cluster, the full manifestation of receptor desensitization may require phosphorylation of more than one of these residues. Our identification of putative residues phosphorylated in spontaneous B2 receptor desensitization is in good agreement with a very recent study of phosphorylated residues in IC-IV of the unstimulated rat B2 receptor using mass spectrometry (17).

The BK-stimulated B2 receptor activity was sensitive to mutation of the same conserved residues as the ligand-independent, spontaneous receptor activity (Fig. 2, right panel). Small but significant increases (1.4–1.8-fold) in BK-stimulated assays were terminated by dilution in ice-cold phosphate-buffered saline, 0.3% bovine serum albumin and rapid vacuum filtration on Whatman GF/C filters previously soaked in 1% polyethyleneimine.

Receptor Activity—Activities of receptor constructs were assayed by monitoring PI hydrolysis and mobilization of intracellular Ca\(^{2+}\) essentially as described previously (13, 15). PI hydrolysis was assayed in HEK293 cells prelabeled with 1 μCi/ml myo-[\(^{3}H\)]inositol in Dulbecco’s modified Eagle’s medium, 5% heat-inactivated horse serum. Following washing, the cells were incubated in Leibovitz’s L-15 medium containing 50 mM LiCl in the absence and presence of ligands for 30 min at 37 °C.

The cytosolic free Ca\(^{2+}\) signal from single fura-2 labeled A10 cells was acquired as the ratio of Ca\(^{2+}\)-bound fura-2/Ca\(^{2+}\)-free fura-2, denoted as F\(_{390}\)/F\(_{380}\), and processed by an integrated digital imaging fluorescence microscopy system. The average of the net peak ratio values for wild-type (WT) and B2ASerThr were 2.44 and 2.15, respectively. Aligning the matching frames from individual traces allowed for the averaging of normalized responses from multiple experiments. As small variations occurred from one experiment to the next in the amount of elapsed time between ligand additions, artificial breaks were inserted in the traces so that both marker frames could be matched.

**RESULTS AND DISCUSSION**

**FIG. 1.** Receptor-mediated stimulation of PI hydrolysis and mobilization of intracellular Ca\(^{2+}\) in cells expressing the human WT B2 receptor and B2ASerThr. A, the amino acid sequence of IC-IV of the human WT B2 receptor is shown. Indicated is the junction of the 7th transmembrane domain and IC-IV (vertical line), serines and threonines (+), and the conserved cluster of two threonines and two serines mutated into alanines to create the receptor construct B2ASerThr (Ser/Thr cluster). B, basal PI hydrolysis was assayed in HEK293 cells expressing varying amounts of the WT B2 receptor (●) or B2ASerThr (○) as indicated. C, PI hydrolysis was assayed in HEK293 cells expressing varying amounts of the WT B2 receptor (●) or B2ASerThr (○, △) in the absence (●, ○) and presence of 1 μM BK (▲, △) as indicated. The effects of BK are shown (dashed arrows). Please note the difference in the y axis scale in B and C. The results are from four to eight independent experiments with each point performed in duplicate. D, intracellular Ca\(^{2+}\) mobilization in A10 cells expressing the WT B2 receptor (●) or B2ASerThr (○) at approximately equal densities (34 ± 5 and 41 ± 12 fmol/10\(^6\) cells, respectively) in the absence and presence of 0.1 μM BK and 1 μM HOE140 as indicated. Traces are averages ± S.E. of single cell Ca\(^{2+}\) traces from 13 cells (two experiments) and 40 cells (three experiments) expressing the WT B2 receptor or B2ASerThr, respectively, which had been normalized by the height of the initial Ca\(^{2+}\) peak.
PI hydrolysis were observed by individually mutating Ser^{339}, Thr^{342}, Thr^{345}, and Ser^{348}, whereas a significantly larger BK stimulation (2.4-fold) was observed by mutating these residues as a cluster. Interestingly, truncation of IC-IV at residue 313 resulted in a construct that bound BK but did not elicit either a spontaneous or BK-stimulated response. In the experiment described in the figure, the alanine mutant of Ser^{316} exhibited an apparently elevated activity, but this activity was due to a higher level of expression of this mutant in this representative experiment. Addition of 1 μM BK to B2A{Ser/Thr} yielded an I_{A2} of 1.5, which was approximately 2-fold greater than that of the WT B2 receptor which was 0.86 (Fig. 1C). The equilibrium dissociation constants of BK binding to the WT B2 receptor (0.104 ± 0.025 nM; n = 2) and B2A{Ser/Thr} (0.123 ± 0.026 nM; n = 2) were approximately the same, indicating that the elevated stimulation of B2A{Ser/Thr} was not due to an increase in the affinity for BK for the receptor. The importance of the Ser/Thr cluster in receptor desensitization was also observed in BK stimulation of intracellular Ca^{2+} mobilization. As shown in Fig. 1D, stimulation of B2A{Ser/Thr} with BK resulted in a response which was biphasic with a first phase characterized by a plateau of elevated free Ca^{2+}, and this response remained elevated for >5 min. That the second phase was due to sustained agonist-stimulated receptor activity was evident from the immediate and large decrease in the free Ca^{2+} level following addition of HOE140 (1 μM) after approximately 5 min of agonist stimulation. Thus, desensitization of ligand-dependent and -independent B2 receptor activity involves the same cluster of serines and threonines in IC-IV. This cluster is located between two acidic residues, Glu^{337} and Glu^{350}, which are conserved among B2 receptors and which have been shown to be important for GRK recognition (18).

Based on the observation of ligand-independent, spontaneous, and mutation-induced GPCR activity, current models state that these receptors spontaneously isomerize between inactive and activated conformational states termed R and R*, respectively, and that R* associates with a G protein to form R*G which triggers the intracellular signal (1, 9). R* is also believed to be a substrate for GRK and, consequently, subject to desensitization (19). In this model, full agonists act by selectively stabilizing, and therefore maximally shifting, the equilibrium toward the activated R* state and, consequently, R*G, whereas inverse agonists act by stabilizing the inactive R state. This model predicts that a full agonist behaves as an agonist independently of the spontaneous receptor activity. The only exception would be if the spontaneous activity is as high as the agonist-stimulated activity in which case the agonist should have no effect. Indeed, BK dose-dependently stimulated the activity of both the WT B2 receptor and B2A{Ser/Thr} (Fig. 3A). Thus, BK acts as an agonist independently of the level of spontaneous activity and receptor desensitization. The higher maximal BK response on B2A{Ser/Thr} was expected, since this construct is subject to less desensitization than the WT B2 receptor (Fig. 3A).

NPC17731 and HOE140 are two structurally similar, potent, and selective BK peptide antagonists (20, 21). Apparently conflicting results have been noted regarding the action of these antagonists. Partial agonism by HOE140 was observed on the rat WT B2 receptor transiently expressed in COS-1 cells (22), whereas inverse agonism by HOE140 and NPC17731 was observed on the native B2 receptor in primary cultures of rat myometrial cells (23). Inverse agonism by HOE140 was also observed on the human WT B2 receptor transiently expressed in COS-7 cells (24). The absence and presence of partial agonism by HOE140 was observed in native B2 receptor preparations from various rabbit and sheep tissues (25). Thus, the efficacy and behavior of HOE140 and/or NPC17731 differ depending on the cell system expressing the B2 receptor. Fig. 3B shows that NPC17731 dose-dependently stimulated the activity of the human WT B2 receptor expressed in HEK293 cells. The same effect was observed with HOE140 (Fig. 3C). Consequently, these ligands act as agonists on this receptor in this cell system. In contrast, NPC17731 and HOE140 dose-dependently inhibited the basal activity of B2A{Ser/Thr} (Fig. 3, B and C). Therefore, these ligands act as inverse agonists on this receptor in this system. These results reconcile the apparently conflicting action of BK antagonists reported in the literature and provide direct evidence that the specific action of NPC17731...
Spontaneous Bradykinin Receptor Activity and Partial Agonism

Spontaneous activity of a spontaneously formed pool of activated R* state.

The action of NPC17731 and HOE140 either as agonists by stimulating B2 receptor activity or as inverse agonists by inhibiting basal receptor activity is in direct conflict with current two receptor state models, as in these models a ligand either favors the active R* state or the inactive R state but not both (1). We previously proposed a three-state model of agonist binding to the B2 receptor, which includes an additional intermediate receptor state (26). This model was based on the ability of BK to promote the sequential formation of three receptor binding states, R ↔ R* ↔ R**, where the formation of the third, equilibrium state R** was blocked by GTP identifying it as the G protein-coupled state of the receptor. Thus, R** in this model equates to R* in two-state models. In the three-state model, the additional intermediate state R* was suggested to be a partially active state of the receptor not yet fully functionally coupled to the G protein (26). This model can explain our results by inferring that NPC17731 and HOE140 stabilize R*. Thus, when the activity of the spontaneously formed R** is low, possibly due to a relatively high level of desensitization, these ligands would reveal themselves as weak agonists by their ability to favor the conversion R → R*. On the other hand, when the activity of the spontaneously formed R** is high due to a relatively low level of desensitization, these ligands would reveal themselves as inverse agonists by their ability to favor the conversion R** → R*. Thus, the intrinsic parameter of these drugs may be their ability to stabilize R*, and drug efficacy and behavior depends on the activity of R* relative to that of R**.

It may be expected that in the above model, the level of maximal stimulation of the WT B2 receptor by NPC17731 and HOE140 should equal that of their maximal inhibition of B2Ado/Thr*. As shown in Fig. 3, B and C, this was not the case. However, this difference may be expected if R* is also a substrate for GRK and, consequently, subject to desensitization. If so, the maximal stimulation of the WT B2 receptor should never reach the maximal inhibition of the less desensitized B2Ado/Thr*.

In conclusion, our results provide several novel and critical pieces of information regarding regulation of ligand-independent GPCR activity and the action of partial receptor agonist. First, these results describe a unique GPCR epitope, which when mutation leads to an increase in spontaneous receptor activity. We believe this mutation is different from previously reported constitutively activating mutations (27), in that it elevates the activity of a spontaneously formed pool of activated receptors by relieving them of desensitization rather than by disrupting interhelical contacts necessary to constrain the receptor in an inactive state. However, an effect of this mutation on the equilibrium constant for the isomerization between the inactive and activated receptor states cannot be excluded. Considering that desensitization varies depending on the cell state and system by variations in, e.g. the activity ratios of receptors and effectors involved in both desensitization and signaling, this mechanism provides the cell with the means to dynamically control GPCR activity independently of a receptor

lend. Second, this mechanism directly influences the action of receptor ligands and, indeed, reconciles the apparent conflict which has been reported in the literature regarding NPC17731 and HOE140. In fact, our results, which directly refute classical pharmacological theories, show that the capacity of a drug to modulate a receptor response is not intrinsic to the drug but is also dependent on the cellular environment in which the drug acts. Chidiac et al. (28) reported that the efficacies of several inverse agonists at the β2-adrenergic receptor were perturbed following agonist pretreatment to induce desensitization. However, the spontaneous receptor activity was not monitored in this study and, consequently, could not be related to the inverse agonist efficacy. Third, our results show unequivocally that inverse agonism is a unique pharmacological parameter rather than the result of antagonism of endogenously released agonist. Fourth, our results are compatible with the existence of a partially activated receptor state which is stabilized by NPC17731 and HOE140 and which is higher in activity than the inactive R state and lower in activity than the activated R* state.

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