Antagonists of Bradykinin That Stabilize a G-protein-uncoupled State of the B2 Receptor Act as Inverse Agonists in Rat Myometrial Cells*

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Several B2 bradykinin (BK) receptor-specific antagonists including HOE140, NPC17731, and NPC567 exhibited negative intrinsic activity, which was observed as a decrease in basal phosphoinositide hydrolysis in primary cultures of rat myometrial cells, and this response was opposite to that elicited by the agonist BK. The order of potency of the antagonists in attenuating basal activity was essentially the same as that in competing both [3H]BK and [3H]NPC17731 for binding to B2 receptors on both intact rat myometrial cells and bovine myometrial membranes. We therefore proposed a three-state model for the binding of agonists to G-protein-coupled B2 receptors in bovine myometrial membranes (Leeb-Lundberg, L. M. F. and Mathis, S. A. (1990) J. Biol. Chem. 265, 9621-9627). This model was based on the ability of BK to promote the sequential formation of three receptor binding states where formation of the third, equilibrium state was blocked by Gpp(NH)p (guanylylimidodiphosphate) identifying it as the G-protein-coupled state of the receptor. Here, we show that, in contrast to BK, these antagonists bound preferentially to a G-protein-uncoupled state of the receptor. These results indicate that B2 receptor antagonists that stabilize a G-protein-uncoupled state of the receptor act as inverse agonists. Furthermore, these results provide strong evidence that endogenous G-protein-coupled receptors exhibit spontaneous activity in their natural environment in the absence of agonist occupancy.

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2 The abbreviations used are: BK, bradykinin; PI, phosphoinositide; DMEM, Dulbecco's modified Eagle's medium; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Gpp(NH)p, guanylylimidodiphosphate; RIA, radioimmunoassay.

Origin (3). PI hydrolysis through activation of phospholipase C represents one of the major intracellular signaling pathways that are mediated by these receptors in various cells (4-7). This response appears to be regulated primarily by a G-protein α subunit of the α, family (8).

A number of B2 receptor peptide antagonists have been developed. A first generation of antagonists with moderate receptor affinities were developed around the crucial replacement of Pro' with a D-aromatic residue (9, 10). Later, a second generation of antagonists were developed specifically to incorporate a conformationally restricted β turn in the C-terminal residues of the BK analogs, which resulted in considerably higher affinities for the B2 receptor (11-15).

Classical drug theories state that agonists either fully or partially stimulate the receptor-mediated functional response, while antagonists block the interaction of the agonists with the receptor but lack any intrinsic activity themselves. Subsequent theories propose that unoccupied receptors can spontaneously isomerize between "inactive" and "activated" states and that antagonists either do not distinguish between these states (neutral antagonists) or they favor the inactive state of the receptor (negative antagonists or inverse agonists) (16, 17).

Inverse agonism was first observed at the benzodiazepine/g-aminobutyric acid type A receptor-CI- ion channel complex where the complex spontaneously isomerizes into an activated conformation in which the channel is open (18). For G-protein-coupled receptor systems, a few similar observations have been made (19), but these have been limited to in vitro systems such as broken cell (20-22) and reconstituted (23, 24) preparations and cell systems transfected with either wild-type (25, 26) or constitutively active receptor mutants (27-30).

Here, we show that several bradykinin antagonists behave as inverse agonists on PI hydrolysis in primary cultures of rat myometrial cells. This activity was correlated with the ability of these antagonists to stabilize a G-protein-uncoupled state of this receptor in bovine myometrial membranes. These results reinforce our previously proposed three-state model for the binding of agonists to the G-protein-coupled B2 receptor (2).

EXPERIMENTAL PROCEDURES

Cell and Membrane Preparations—Primary cultures of rat myometrial cells and bovine myometrial membranes were prepared as described by Tropet et al. (7) and Leeb-Lundberg and Mathis (2), respectively. Protein concentration was determined by the method of Bradford (31) using bovine serum albumin as a standard.

Receptor Binding—Radioligand binding assays with the agonist [3H]BK and the antagonist [3H]NPC17731 were performed essentially as described (2, 7). In short, intact cell binding was assayed in DMEM, 0.1% bovine serum albumin including the protease inhibitors bacitracin (140 μg/ml) and 1,10-phenanthroline (1 μM) at 4°C for 90 min. Membrane binding was assayed in 25 mM TES, pH 6.8, 0.5 mM EDTA, 1 mM MgCl2 including the above inhibitors, at 25°C for 60 min. In both protocols, nonspecific binding was determined in the presence of 1 μM BK.

In some experiments, any receptor-bound BK was removed prior to binding assays on intact cells by incubating cells with a low pH buffer (50 mM glycine-HCl, pH 3.0) for 3 min at 4°C followed by two 1-ml washes with phosphate-buffered saline containing 0.3% bovine serum albumin as described by Munoz and Leeb-Lundberg (32) and Munoz et al. (33).

PI Hydrolysis—Cells were assayed essentially as described (7), with a few modifications. Briefly, confluent cells were incubated with 10 μCi/ml 3H[GABA] in DMEM, 1% heat-inactivated fetal bovine serum at 37°C for 24 h in 10% CO2. Prior to experimentation, the cells were washed four times with 1 ml of DMEM and incubated in DMEM, 50 mM L-Cl for 30 min. Following replacement with 2 ml of the same medium, the cells were incubated at 37°C with various agonists and

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antagonists for 20 min. Inositol phosphates were then extracted and isolated using anion exchange chromatography.

Analysis of Cell-derived BK—Medium (40 ml) incubated as described above for PI hydrolysis and low pH buffer wash of incubated cells were supplemented with ~10^6 dpm (~58 pg) [^3H]BK (internal standard). The supplemented preparations were then processed on a C18 SepPak cartridge (Waters Associates). The recovery of[^3H]BK was ~90%. The BK concentration was then assayed by RIA according to a procedure described by the supplier (Peninsula, Belmont, CA). Identification of the internal standard in the RIA confirmed absence of BK degradation during preparation.

Materials—[^2,3,3H]Bradykinin (82.3–110 Ci/mmol) and [prolyl-3,4-[^3H]NPKC17731 (53.5 Ci/mmol) were purchased from DuPont NEN, and myo[^3H]inositol (17 Ci/mmol) was purchased from American Corp. Timed-pregnant Sprague-Dawley rats were from Harlan (Indianapolis, IN), and fresh uteri from pregnant cows were from a local slaughterhouse. HOE140 and NPC567 were from Bachem, Inc. (Torrance, CA). BK was purchased from Sigma. Gpp(NH)p was from Boehringer Mannheim. A BK RIA kit was purchased from Peninsula. NPC17731 was the kind gift of Dr. S. Farmer of Zeneca Pharmaceuticals Group (Wilmington, DE).

RESULTS AND DISCUSSION

Primary cultures of rat myometrial cells contain a large number of a single type of B2 receptor as determined by the high affinity specific binding of the agonist[^3H]BK (185,000 ± 47,000 sites/cell; K_d = 1.8 ± 0.2 nM; n = 3) (7). Fig. 1A shows that this binding was specifically competed by the antagonists HOE140, NPC17731, and NPC567 with an order of potency typical for a B2 receptor. The antagonist[^3H]NPC17731 identified the same B2 receptor as determined by the amount (230,000 ± 5,000 sites/cell; n = 3) and the affinity (K_d = 0.9 ± 0.2 nM) of binding as well as by the specificity of agonist and antagonist competition (Fig. 1B).

Addition of BK to rat myometrial cells resulted in a dramatic and dose-dependent increase in PI hydrolysis indicating that BK acts as a full agonist on these cells (7) (Fig. 2, upper panel). This response was clearly mediated through the B2 receptor as it was inhibited by the above antagonists with an appropriate order of potency but not by des-Arg[Leu]^8^BK, a B1 receptor antagonist (data not shown); furthermore, the EC_{50} value (3.7 nM) of BK stimulation was very similar to the IC_{50} values (1.7 nM; see Fig. 1) for BK in competing for both[^3H]BK and[^3H]NPC17731 binding.

Interestingly, each of these antagonists exhibited negative intrinsic activity on their own (in the absence of agonist) by significantly reducing the basal level of PI hydrolysis (Fig. 2, lower panel). As expected for receptor-mediated events, these reductions were dose-dependent and saturable. Furthermore, the maximal reduction (35–40%) in the basal PI hydrolysis was virtually the same for all the antagonists, and the potency of each in reducing the basal activity was similar to their potency in competing for both[^3H]BK and[^3H]NPC17731 binding to these cells. By exhibiting negative intrinsic activity, these antagonists appear to behave as inverse agonists such as have been observed with antagonists in some ion channel receptor systems (18).

An alternative explanation for the decrease in basal activity is simple antagonism of stimulation by cell-derived BK released during the assay. As shown by the dose-response curve in Fig. 2, a minimum of 0.1 nM BK was required to elevate PI hydrolysis in these cells. By RIA, which is sensitive to as little as 0.25 pg, no BK was detected in medium previously incubated with cells as described for the assay of PI hydrolysis (conditioned medium). The same result was obtained from RIA of collected low pH buffer used to strip any bound cell-derived BK from the extracellular surface of incubated cells. Furthermore, conditioned medium did not inhibit either[^3H]BK or[^3H]NPC17731 binding to naive cells. In addition, we found no increase in the binding of either radioligand to incubated cells that had been stripped with the low pH buffer (data not shown). Thus, the amount, if any, of cell-derived BK in the medium during the functional assay is <0.25% of that required for stimulation of PI hydrolysis. Consequently, the negative intrinsic activity of these antagonists cannot be explained by inhibition of the action of cell-derived BK.

Inverse agonism requires that the unoccupied B2 receptor spontaneously isomerizes between an inactive conformational state, which can be stabilized by antagonists, and an activated state, stabilized by agonists. Previously, we proposed a three-state model for BK binding to GTP-sensitive B2 receptors in bovine myometrial membranes (Fig. 3) (2). According to this model, BK (L) and receptor (R) initially form a complex from...
which BK dissociates rapidly, which is termed LR. In a time-
dependent manner, the binding complex becomes stabilized
through the conversion of LR through LR' to a state from which
BK dissociates very slowly, termed LR**. Inclusion of Gpp-
(NH)p, a nonhydrolyzable analog of GTP, completely blocks the
formation of LR**. We believe LR* is the state in which the
receptor exists while functionally coupled with the G-protein,
thus, it is termed LR"G. BK dissociates more slowly from LR"G
than from either R or R'. Thus, just as agonist receptor binding
promotes increased G-protein-receptor coupling, G-protein cou-
pling apparently results in increased agonist affinity. Unlike
the ternary complex model described by DeLean et al. (34) to
explain the interaction of agonists and antagonists with G-
protein-coupled beta-adrenergic receptors, our model contains the
additional receptor state R' to which the agonist can be bound.
Considering the relative position of R' in the binding sequence,
we originally suggested that this state represents a "pre-
coupled" or activated form of the receptor not yet functionally
coupled to the G-protein (2). Conceptually, our model derived
from studies in bovine myometrial membranes can accommo-
date the inverse agonism observed in rat myometrial cells by
equating R with the antagonist-stabilized inactive receptor
state and R' with the agonist-stabilized activated receptor
state.

We compared the pharmacological characteristics of agonist
and antagonist binding in these two receptor systems. As in rat
myometrial cells, [3H]BK and [3H]NPC17731 identified an
equal number of binding sites in the bovine membranes. Fig. 4
(A-G) shows that the order of potency of BK, HOE140,
NPC17731, and NPC567 in competing for [3H]BK binding was
virtually the same as competing for [3H]NPC17731 binding,
indicating that these two radioligands identify the same recep-
tor in these membranes. Furthermore, the specificity of agonist
and antagonist binding in rat myometrial cells and bovine myo-
metrial membranes was also very similar (compare Figs. 1 and
4), indicating that these two systems contain B2 receptors that
are at least pharmacologically virtually identical.

Since the agonists bind with higher affinity to the G-protein-
coupled state of the receptor, in order to exhibit negative in-
trinsinc activity inverse agonists must bind preferentially to
a G-protein-uncoupled state. To demonstrate this experiment-
ally, we compared agonist and antagonist binding in bovine
membranes in the absence and presence of Gpp(NH)p. Inclu-
sion of 10 μM Gpp(NH)p resulted in a rightward shift (6.3-fold)
in the competition curve for BK when competing for
[3H]NPC17731 binding (Fig. 4A). This classical result agrees
with our model and with our functional results, all of which
indicate that agonists prefer to bind to and favor the forma-
tion of the G-protein-coupled state of the receptor. No Gpp(NH)p-
induced shift was observed in the competition curve for BK

\[ K_{d1} \neq K_{d2} \neq K_{d3} \]

when competing [3H]BK binding (Fig. 4D). This observation is
not surprising since BK binds in a manner identical to that of
[3H]BK and, consequently, is subject to the same heterogeneity
in the binding affinity to different conformational states of the
receptor. The same reasoning may be used in interpreting the
absence of a Gpp(NH)p-induced shift in the competition curves
for the antagonists HOE140 (Fig. 4B) and NPC17731 (Fig. 4C)
when competing [3H]NPC17731 binding. In contrast, when com-
peting for [3H]BK binding, Gpp(NH)p induced a leftward
shift (3.8-fold; Fig. 4E), NPC17731 (5.2-fold; Fig. 4F), and NPC567 (1.5-fold; Fig. 4G). These results indicate that agonists and antagonists prefer to bind to different states of the receptor. The effect of Gpp(NH)p shows that the antagonists stabilize a G-protein-uncoupled state.

This report describes several important observations that
directly relate to the nature of the different agonist binding
states of the B2 receptor (2). Based on our observation of B2
receptor antagonists with negative intrinsic activity, we con-
clude that, as in some other receptor systems (18), this receptor
can spontaneously isomerize between an inactive and an acti-
vated conformational state in the absence of agonist binding
and that when the receptor is in the activated state, it can
couple with a G-protein to trigger a functional response. Re-

tults on which our model was originally based indicate that
only after agonist binding for very short time periods (seconds)
does R represent the major receptor state (2). On the other
hand, at equilibrium agonist binding, in the presence of Gp-
(NH)p, which prevents R' from isomerizing into R"G, R"G
represents over 50% of the total agonist binding. Thus, over time
agonist binding clearly favors formation of R"G at the expense of
R. In the absence of Gpp(NH)p, the agonist rapidly converts R"G
back to R, the G-protein-coupled state of the receptor. Conse-

quently, we conclude that R represents the inactive state of the
receptor and R"G represents the agonist-stabilized activated
receptor state, which is not yet functionally coupled to the G-

protein.

\[ \text{FIG. 3. Proposed agonist binding model of the G-protein-
coupled B2 bradykinin receptor in bovine myometrial mem-
}

\[ \text{branes. The model has been described in detail previously (2). } K_x, K_y,
\text{and } K_z \text{ represent equilibrium association constants describing the bind-
}\]

\[ \text{ing of agonist (L) to G-protein (G)-uncoupled and -coupled receptor (R),
}\]

\[ \text{and } K_x, K_y, K_z, \text{ and } K_4 \text{ represent unimolecular constants describing the
}\]

\[ \text{isomerization } R \rightarrow R' \rightarrow R"G \text{ with and without bound L.}
\]

\[ \text{FIG. 4. Agonist and antagonist competition binding curves in the
}\]

\[ \text{absence and presence of Gpp(NH)p in bovine myometrial mem-
}\]

\[ \text{branes. Membranes were incubated with a constant concentration of
}\]

\[ [3H]NPC17731 (1 \text{ nmol/L}) \text{ and [3H]BK (1 \text{ nmol/L}) and with increasing
}\]

\[ \text{concentrations of BK (A and D), HOE140 (B and E), NPC17731 (C and F),
}\]

\[ \text{or NPC567 (G) in the absence (open symbols) and presence of 10 \text{ μM Gpp(NH)p (closed symbols) as described under "Ex-
}\]

\[ \text{perimental Procedures." The results are presented as percent of control,
}\]

\[ \text{where control refers to specific [3H]NPC17731 and [3H]BK binding to
}\]

\[ \text{membranes (80–100 μg of protein/10 μl of assay) as determined in the
}\]

\[ \text{presence of 1 μM BK. 100% Control represents 469 ± 48 \text{ (Gpp(NH)p-
}\]

\[ \text{and 450 ± 85 fmol/mg of protein (Gpp(NH)p) \text{ of [3H]NPC17731 binding
}\]

\[ \text{534 ± 62 (Gpp(NH)p) and 450 ± 85 fmol/mg of protein (Gpp-
}\]

\[ \text{(NH)p) of [3H]BK binding, respectively. The results shown in panels A–F
}\]

\[ \text{are the averages ± S.E. of two experiments, and panel G shows the
}\]

\[ \text{result of two experiments. Each point was performed in duplicate.
}\]

\[ \text{This observation is not surprising since BK binds in a manner identical to that of
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receptor and R"G represents the agonist-stabilized activated
receptor state, which is not yet functionally coupled to the G-

protein.
As predicted by the model for a ligand that stabilizes the inactive R state, addition of Gpp(NH)p to block the agonist-promoted formation of R*G and shift the equilibrium to the inactive R state increased the affinity of the antagonists for the receptor (Fig. 4). This antagonist selectivity is further supported by the fact that addition of Gpp(NH)p in the absence of any agonist had no significant effect on the dissociation rate of [3H]NPC17731 binding (Fig. 5). Even though these antagonists inhibit BK binding, cross-linking studies indicate that the binding sites for NPC17731 and HOE140 in the B2 receptor are not identical to that for BK, providing one possible explanation as to why these two classes of ligands stabilize different conformational states of the receptor.

In order to accommodate the observations of constitutive activity for some G-protein-coupled receptors in membranes and reconstituted systems (20–24) and in transfected systems using either wild-type (25, 26) or constitutively active receptor mutants (27–30), an extension of the original ternary complex model was recently reported by Samama et al. (35), and this model accommodates inverse agonism (30). Even though described in considerably more detail, the model proposed by Samama et al. is similar if not identical to the model described by us previously (2) and elaborated upon in this report. The resemblance between these two models suggests that G-protein-coupled receptors behave similarly regardless of whether they accept peptidergic or non-peptidergic ligands.

In general, second generation B2 receptor antagonists, of which HOE140 and NPC17731 are representative examples, are at least 10-fold more potent in antagonizing BK-stimulated functional responses than first generation antagonists, of which NPC567 is a representative example (36). This difference in agonistic potency was not directly reflected in the potency of these antagonists to compete for [3H]BHK binding on the myometrial preparations where NPC567 was about equi-potent with HOE140 and NPC17731. In contrast, NPC567 was approximately 16- to 13-fold less potent than HOE140 and NPC17731, respectively, in inhibiting basal PI hydrolysis. Furthermore, while the Gpp(NH)p-induced shift of the NPC567 competition curve was 1.5-fold, the HOE140 and NPC17731 curves were shifted 3.8- and 5.2-fold, respectively. Thus, the ability of antagonists to stabilize the inactive conformation of the B2 receptor is a more accurate parameter of antagonistic activity in this system than their ability to displace BK binding to the receptor.

In summary, the results reported here serve to further define the nature of each of the binding states in our model. This was possible due to 1) B2 receptor ligands exhibiting negative intrinsic activity, 2) identification of a cell system with a sufficiently high number of B2 receptors that exist spontaneously in an activated state, and 3) the relatively high affinity binding of BK to both G-protein-coupled and -uncoupled states of the B2 receptor. The few previous studies with other G-protein-coupled receptors in intact cells have all used cells transfected with either wild-type or constitutively activated mutant receptors. To our knowledge, this is the first study demonstrating in a primary cultured cell system containing an endogenous G-protein-coupled receptor that inverse agonism in this super-family of receptors is not an artifact but a natural feature of the receptor system, and this receptor behavior is supported by our proposed model.

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