Na^+ Ions Binding to the Bradykinin B_2 Receptor Suppress Agonist-Independent Receptor Activation†

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ABSTRACT: Control of the balance between receptor activation and inactivation is a prerequisite for seven transmembrane domain (7TM) receptor function. We asked for a mechanism to stabilize the inactive receptor conformation which prevents agonist-independent receptor activation. Na^+ ions have reciprocal effects on agonist versus antagonist interaction with various 7TM receptors. To investigate the Na^+ dependence of receptor activation we chose the bradykinin B_2 receptor as a prototypic 7TM receptor. Decrease of the intracellular Na^+ content from 40 mM to 10 mM of COS-1 cells transiently expressing rat B_2 receptors activated the B_2 receptor in the absence of agonist as shown by a 3-fold increase in the basal release of inositolphosphates and increased the intrinsic activity of bradykinin to 1.2. In contrast, under increased intracellular Na^+ (148 mM) the intrinsic activity of bradykinin decreased to 0.72. When the interaction of Na^+ with the B_2 receptor was prevented by exchanging a conserved aspartate in transmembrane domain II for asparagine the B_2 receptor was also constitutively-activated in the absence of agonist. Agonist-independent B_2 receptor activation under decreased intracellular Na^+ was similarly observed with primary human fibroblasts endogenously expressing human B_2 receptors by a 2.5-fold increase in basal inositolphosphates. Activation of human B_2 receptors in the absence of agonist under decreased intracellular Na^+ was further evident by an increased basal phosphorylation of the B_2 receptor protein. Thus our data suggest that the interaction of Na^+ ions with the B_2 receptor stabilizes or induces an inactive receptor conformation thereby providing a mechanism to suppress agonist-independent receptor activation *in vivo*.

A feature of several 7TM[1] receptors known long before the cDNA sequences of the receptors have been cloned is the reciprocal modulation of agonist binding versus antagonist binding by monovalent cations (Pert & Snyder, 1974; Tsai & Lefkowitz, 1978). Several observations suggest that an aspartate (Horstman et al., 1990) at the intracellular edge of transmembrane domain-II (TM-II) which is highly conserved among G protein-coupled receptors (Baldwin, 1993) is at least in part responsible for this effect. The biological function of this "Na^+ effect", however, is not clear (Motulsky & Insel, 1983; Ceresa & Limbird, 1994). Therefore we investigated the role of Na^+ binding on a member of the 7TM receptor family and chose the bradykinin B_2 receptor as our model system. Binding of the peptide hormone bradykinin to the B_2 receptor activates a $G\alpha_q$ family-mediated signal transduction cascade (Jones et al., 1995). Mono- and divalent cations are reported to decrease the number of binding sites for [^{3}H]bradykinin (Roscher et al., 1983). The protein sequences of the rat and human B_2 receptor revealed the presence of the conserved aspartate within TM-II at position 78 or 76, respectively (McEachern et al., 1991; Hess et al., 1992). Therefore the B_2 receptor seemed appropriate for the analysis of the effect of monovalent cations on agonist binding and receptor activation within a living cell. Here we present that intracellular Na^+ ions dose-dependently stabilize or induce an inactive B_2 receptor conformation. This effect of Na^+ ions may be important to suppress agonist-independent receptor activation *in vivo*.

MATERIALS AND METHODS

Materials. Dowex AG1 × 8 was from Sigma; Lipofect-Amine was from Gibco; [prolyl^{2,3}-3,4(*n*)-^{3}H]bradykinin (specific activity 76 Ci/mmol) and myo-[2-^{3}H]inositol (specific activity 17 Ci/mmol) were from Amersham; catalytic hydrogenation with tritium gas of 2-[3,4-dehydro]prolyl^5-[4-iodo]phenylalanyl HOE140 yielded [^{3}H]Phe^{5}HOE-140, and the product was HPLC-purified and characterized by HPLC and mass spectrometry as to its chemical purity (96%), identity, and specific activity (56 Ci/mmol).

Mutagenesis of the Rat B_2 Receptor. The rat B_2 receptor mutant was made by a modification of the PCR mutagenesis method (Freedman et al., 1992). DNA was transiently expressed in COS-1 cells using the expression plasmid pSRF-159, which is a derivative of the SRα promotor vector pcDLSRa296 (Takebe et al., 1988). The mutagenesis was performed on a cassette encompassing the unique *Xba*I and

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[1] Abbreviations: 7TM receptor, seven transmembrane domain receptor; TM-II, transmembrane domain-II; HEPES, (*N*-[2-hydroxyethyl)-piperazine-*N*′-[ethanesulfonic acid]); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CHAPS, 3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonate; BBA-Lys^{0}HOE140, (4-benzoyl)benzoic acid-Lys^{0}HOE140.

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*Pvu*II sites of the protein coding region. The mutagenesis cassette was completely sequenced on an ABI-373A sequencer using the dye terminator method to confirm the identity of the mutant.

Cell Culture and Cell Transfection. COS-1 cells were maintained in RPMI 1640 medium and human foreskin fibroblasts, HF-15 cells (Roscher et al., 1983) endogenously expressing 0.5−1 pmol of B_2 receptors/mg of protein were grown in DMEM and used in passages 7−10. Both media were supplemented with 10% (v/v) fetal calf serum, and cells were kept in a humidified 95% air/5% CO_2 atmosphere. COS-1 cells were transfected using Lipofectamine (Gibco) according to the manufacturer's instructions with varying amounts of the expression plasmid pSRF-159 to obtain the indicated B_2 receptor expression levels. Forty-eight hours after transfection cells were assayed for binding or inositol phosphate release. Membrane preparation was done as previously described (AbdAlla et al., 1996a).

Saturation and Competition Binding. Saturation binding was performed at 4 °C with increasing concentrations (1×10^{-12} to 2×10^{-8} M) of [^{3}H]Phe^{5}HOE140 (specific activity 56 Ci/mmol) or of [^{3}H]bradykinin (specific activity 72 Ci/mmol) on membranes of COS cells suspended in ice-cold binding buffer (10 mM Na^+-HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM KCl, 138 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1 μM leupeptin, 2 μM enalaprilate, 1 mM bacitracin). Where indicated NaCl was substituted by 138 mM *N*-methyl-D-glucamine chloride ("low-Na^+" buffer) or was omitted (buffer of low ionic strength). Nonspecific binding was determined in the presence of 10 μM cold ligand. It was less than 2% of total binding. For competition binding 1 nM [^{3}H]Phe^{5}HOE140 was incubated for 2 h in the presence of increasing concentrations of bradykinin or of HOE140 (1 pM to 10 μM). After 2 h bound ligand was separated from free ligand by rapid filtration over Whatman GF/C filters pretreated with 0.3% polyethyleneimine. Saturation and competition binding data were analyzed using the EBDA/Ligand program (Munson & Rodbard, 1980). Determinations were performed in triplicate (± standard error), and K_D or K_i values are the means (± standard error) of at least three independent experiments.

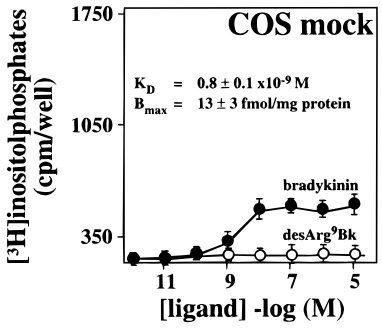
Determination of Total Inositolphosphates. For determination of inositolphosphates adherent COS-1 or HF-15 cells grown on 24-well plates were labeled with myo-[2-^{3}H]-inositol (1 μCi/ml, specific activity 17 Ci/mmol) for 24 h in inositol-free Dulbecco's modified Eagle's medium. Prior to the experiment cells were incubated for 15 min in incubation buffer (138 mM NaCl, 5 mM KCl, 10 mM LiCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Na^+-HEPES, pH 7.2) or in incubation buffer where 138 mM Na^+ had been substituted by 138 mM *N*-methyl-D-glucamine. Then buffer alone or ligands were added to the cells. After 15 min total inositolphosphates were extracted by additon of 20% perchloric acid. Extracts were neutralized with 1 M KOH, and precipitates were removed by centrifugation. Supernatants were applied to anion exchange columns (DowexAG1 × 8), and total inositolphosphates were eluted by 1 M HCl (McCann et al., 1989). Determinations were performed in triplicate (± standard error), and EC_{50} values are the means (± standard error) of three independent experiments.

Identification of B_2 Receptors by Anti-Peptide Antibodies in Immunoblotting. COS cells transiently expressing rat B_2 receptors or mock-transfected COS cells were solubilized by 20 mM 3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonate (CHAPS) in 150 mM NaCl, 50 mM Tris, 5 mM EDTA pH, 7.4 including 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/mL, 1 μM E64, 1 μg of pepstatin/mL. Residual cell debris was removed by centrifugation (100 000*g*, 30 min, 4 °C). Proteins were acetone-precipitated, separated by SDS−PAGE (Laemmli, 1970), and transferred to nitrocellulose using the semidry blotting method (Kyhse-Anderson, 1984). Blots were probed for B_2 receptors using a mixture of domain-specific antibodies raised to the different extracellular domains of the B_2 receptor as described previously (AbdAlla et al., 1996a). Bound antibodies were visualized using the chemiluminescence detection method (ECL kit, Amersham).

Photoaffinity-Labeling of Human B_2 Receptors. Human foreskin fibroblasts (1×10^6 cells) were washed twice with ice-cold phosphate-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells suspended in PBS with protease inhibitors (see above) were incubated in the dark for 1 h at 4 °C with 5 nM (4-benzoyl)benzoic acid-Lys^{0}HOE140 (BBA-Lys^{0}HOE140) in the presence or absence of a 1000-fold molar excess of bradykinin. The photoreactive group of BBA-Lys^{0}HOE140 was then activated by irradiation for 15 min at a wavelength of 365 nm. The cells were rapidly washed with PBS and solubilized by 20 mM CHAPS in 150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4, including protease inhibitors (10 μg of leupeptin/mL, 1 mM phenylmethylsulfonyl fluoride, 1 μM E64, 1 μg of pepstatin/mL). Cell debris was separated from the solubilized proteins by centrifugation (100 000*g*, 30 min, 4 °C). Proteins were precipitated by acetone, separated by SDS−PAGE, and transferred to nitrocellulose sheets. BBA-Lys^{0}HOE140-labeled B_2 receptors were detected by polyclonal or monoclonal anti-HOE140 antibodies as described previously (AbdAlla et al., 1996a).

Whole Cell Phosphorylation. Adherent HF-15 cells expressing 0.5−1 pmol of B_2 receptors/mg of protein were grown on 50 mm diameter dishes. Cells were washed three times with phosphate-free medium and incubated with phosphate-free Dulbecco's modified Eagle's medium without serum for 2 h. Then the cells were labeled with [^{32}P]-orthophosphate (0.1 mCi/ml) for 12 h. Cells were washed twice with incubation buffer or with buffer in which 138 mM Na^+ had been substituted by 138 mM *N*-methyl-D-glucamine. The indicated buffer with 0.1 mCi of [^{32}P]-orthophosphate/mL was then added for 10 min prior to addition of 500 nM bradykinin, 5 μM HOE140, or buffer as a control. After stimulation for 5 min at 37 °C, medium was suctioned and cells were rapidly washed three times with ice-cold medium. The cells were solubilized by 0.2 mL buffer A (150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) with 20 mM 3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonate CHAPS and with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/mL, 1 μM E64, 1 μg of pepstatin/mL, 10 μM okadaic acid, 10 mM NaF, 10 mM disodium pyrophosphate). The solubilisate was centrifuged at 100 000*g* to remove residual cell debris and the CHAPS was diluted to 4 mM by addition of buffer A. B_2 receptors were immunoprecipitated by a mixture of antibodies to the carboxy terminal and the different extracellular domains of the B_2 receptor (AbdAlla et al., 1996a) which were bound to protein G sepharose (50 μL of gel/5 μL of antiserum). After a 2 h incubation at 20 °C and

A



B

rat B_2 receptor	[^{3}H]Phe^{5}HOE140 K_D (nM)	[^{3}H]bradykinin K_D (nM)
wild-type	0.4 ± 0.05	0.4 ± 0.05
D78N	0.5 ± 0.1	0.3 ± 0.1

FIGURE 1: (A) Release of inositolphosphates of COS-1 cells. Total inositolphosphates of mock-transfected COS-1 cells were determined after stimulation by bradykinin (●) or by desArg^{9}bradykinin (○) applied in concentrations between 1×10^{-12} and 1×10^{-5} M. Experiments were performed in triplicate (± standard error); the EC_{50} values are the means (± standard error) of three independent experiments. (B) Affinity of [^{3}H]bradykinin and [^{3}H]Phe^{5}HOE140 for wild-type and D78N-mutated B_2 receptors. Binding of bradykinin or HOE140 to membranes of COS cells transiently expressing wild-type or mutated (D78N) B_2 receptors were assayed 48 h after transfection. Saturation binding was performed for 2 h at 4 °C with increasing concentrations (1×10^{-12} to 2×10^{-8} M) of [^{3}H]-labeled ligand in buffer of low ionic strength without Na^+. Data are the means of three independent experiments (± standard error) each performed in triplicate.

extensive washing with buffer A, bound proteins were recovered by boiling the sepharose in 50 μL of SDS sample buffer. Proteins were separated by SDS−PAGE under reducing conditions. The [^{32}P]-labeled B_2 receptors were visualized by autoradiography. Results presented were similarly obtained in three independent experiments.

RESULTS

Characterization of B_2 Receptors Endogenously Expressed on COS-1 Cells. To analyze the effect of Na^+ ions on the binding of B_2 agonists and antagonists and on the activation of B_2 receptors we chose transient expression of B_2 receptors in COS-1 cells. We first asked whether COS-1 cells display all effectors necessary to functionally couple B_2 receptors to a $G\alpha_q$ family-mediated signal transduction cascade. Addition of bradykinin to mock-transfected COS-1 cells revealed the presence of endogenously expressed B_2 receptors in this cell line (Figure 1A). The potency of bradykinin indicated by the concentration of bradykinin to produce the half-maximum release of inositolphosphates (EC_{50}) was (5 ± 0.5) $\times 10^{-9}$ M. A B_1 receptor agonist, desArg^{9}bradykinin (Regoli & Barabé, 1988), did not induce a significant release of inositolphosphates at concentrations up to 10 μM, indicating that the bradykinin-evoked signal on COS cells is specifically mediated by the activation of B_2 receptors and that COS cells do not express significant amounts of functional B_1 receptors (Figure 1A). This B_2 receptor classification was further supported by the finding that the B_2 antagonist HOE140 (1 μM) suppressed the release of inositolphosphates on COS cells evoked by 10 nM bradykinin (not shown). The amount of endogenously expressed B_2 receptors was 13 ± 3 fmol/mg of protein as determined by saturation binding with the B_2 antagonist [^{3}H]Phe^{5}HOE140 (Wirth et al., 1991). Thus COS-1 cells similarly to COS-7 cells (Quitterer et al., 1995) endogenously express minute amounts of functional B_2 receptors and all effectors necessary for the functional coupling of B_2 receptors to a phospholipase C-mediated second messenger cascade.

Binding of [^{3}H]Bradykinin and [^{3}H]Phe^{5}HOE140 in the Presence of Different Concentrations of Na^+ Ions. Na^+ ions are reported to have reciprocal effects on agonist versus antagonist binding of various 7TM receptors. To compare potential effects of Na^+ on B_2 agonist and antagonist binding we first asked whether the B_2 agonist bradykinin and the B_2 antagonist HOE140 recognize the same number of binding sites. We determined the affinity and the number of binding sites on membranes of B_2 receptor-transfected COS-1 cells. In nominally Na^+-free buffer with low ionic strength to suppress ionic effects on ligand binding [^{3}H]bradykinin and [^{3}H]Phe^{5}HOE140 each bound to the same number of binding sites with a $K_D = (0.4 \pm 0.1) \times 10^{-9}$ M (Figure 1B). Scatchard transformation of the saturation binding data revealed the presence of a single population of binding sites (not shown). These data confirm (Wirth et al., 1991) that bradykinin and HOE140 selectively bind to a single binding site on the B_2 receptor.

Next we analyzed whether Na^+ ions affect the binding of the agonist [^{3}H]bradykinin to B_2 receptors expressed in COS cells. We performed saturation binding with [^{3}H]bradykinin (1×10^{-12} to 1×10^{-8} M) in the presence of increasing Na^+ concentrations. Increasing the Na^+ concentration from 10 mM to 1 M resulted in an apparent decrease of the total number of binding sites for the agonist [^{3}H]bradykinin from 14 pmol/mg of protein to 30 fmol (Figure 2A, left panel). The affinity of bradykinin to the residual B_2 receptor binding sites was not altered, $K_D = (0.4 \pm 0.1) \times 10^{-9}$ M. The number of B_2 binding sites determined for the B_2 antagonist [^{3}H]Phe^{5}HOE140 slightly increased from 14.5 pmol/mg of protein to 18 pmol/mg of protein in the presence of Na^+ ions (Figure 2A, left panel).

Since Na^+ ions did not decrease the receptor number for the B_2 antagonist the apparent decrease in the number of binding sites for the agonist could not result from a loss of receptors. We therefore determined the affinity of bradykinin in the presence of 1 M Na^+ by competition binding with HOE140 and compared it with the affinity determined in the presence of 10 mM Na^+. This was feasible since the binding of HOE140 and bradykinin to rat B_2 receptors expressed in COS-1 cells is mutually competitive (unpublished observation). The concentration of 1 M Na^+ was chosen since it had the maximum effect on agonist binding (cf. Figure 2A). In the presence of 1 M Na^+ the affinity of bradykinin decreased 100-fold, the K_i was (1 ± 0.1) $\times 10^{-9}$ M in buffer with 10 mM Na^+, and (1 ± 0.2) $\times 10^{-7}$ M (Figure 2B-I) in the presence of 1 M Na^+. We conclude that the apparent decrease of the number of bradykinin binding sites in the presence of increasing concentrations of Na^+ ions (cf. Figure 2A) is due to a 100-fold decrease in agonist affinity. The affinity of the partial agonist HOE140 decreased 2-fold, and the K_i was (0.5 ± 0.1) $\times 10^{-9}$ M in "low-Na^+" buffer and (1.1 ± 0.2) $\times 10^{-9}$ M in "high-Na^+" buffer, respectively (Figure 2B-III). Though various 7TM receptors undergo an affinity shift of their respective agonists in the presence of Na^+ [for overview see Ceresa & Limbird, (1994)], the 100-fold decrease in the affinity of the B_2 agonist

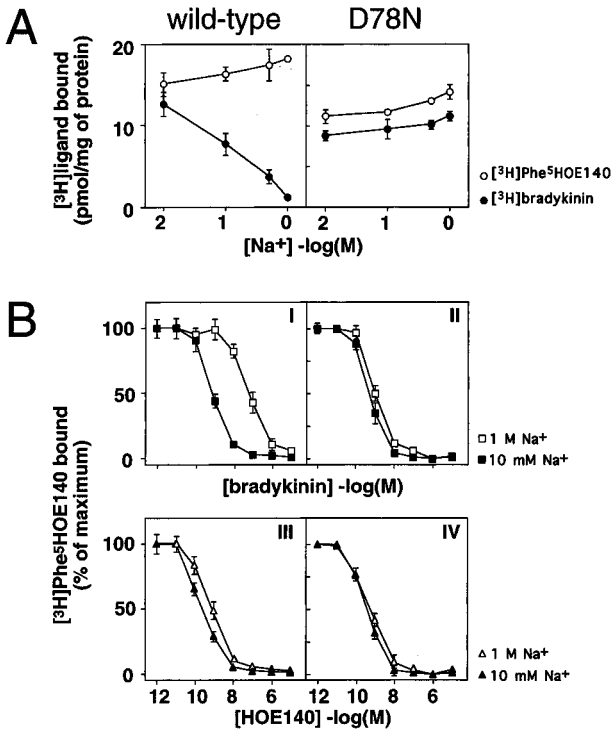


FIGURE 2: (A) Binding of bradykinin or HOE140 in the presence of various concentrations of Na^+. COS-1 cells were transfected with cDNAs of the wild-type rat B_2 receptor or of the D78N mutant, and the apparent number of binding sites for [^{3}H]bradykinin (●) or for [^{3}H]Phe^{5}HOE140 (○) was determined in the presence of increasing concentrations of Na^+ ions. In buffer with a Na^+ concentration of 0.1 and 0.01 M, NaCl was substituted by *N*-methyl-D-glucamine-chloride to maintain an ion concentration of 0.5 M. (B) Competition binding in the presence of 1 M, and 0.01 M NaCl. Binding of 1 nM [^{3}H]Phe^{5}HOE140 to membranes of COS-1 cells expressing wild-type receptor (I, III) or of cells expressing the D78N mutant (II, IV) was determined in the presence of increasing concentrations of bradykinin (I, II) or of HOE140 (III, IV) in binding buffer containing 0.01 M (■, ▲) or 1 M (□, △) Na^+. Data are expressed as % of maximum binding determined in the absence of competitor. Experiments were performed in triplicate (± standard error), and K_i values are the means (± standard error) of three independent experiments.

bradykinin in the presence of Na^+ ions is unprecedented and may reflect the high sensitivity of B_2 receptors to the presence of Na^+ ions.

Effect of Na^+ Ions on Ligand Binding to the B_2 Receptor Mutant D78N. Other workers have shown for, e.g., dopamine D_2 (Neve et al., 1993) or α_2-adrenergic (Horstman et al., 1990) receptors that a major residue mediating a sodium effect is the highly conserved aspartate of the LAXAD motif of TM-II. Thus we examined the effect of mutating aspartate 78, the conserved aspartate in TM-II of the rat B_2 receptor, to asparagine. Exchange of aspartate 78 for asparagine did not influence the affinity of bradykinin or HOE140 in "low Na^+ buffer" (Figure 1B and 2B-II/IV). In addition, when aspartate 78 of the B_2 receptor was exchanged for asparagine the presence of Na^+ did not decrease the apparent number of agonist binding sites (Figure 2A, right panel), and the concentration of 1 M Na^+ did not decrease the affinity of the agonist or antagonist (Figure 2B-II,IV). Together the preceding findings strongly indicate that Na^+ ions dose-dependently bind to the negative charge of aspartate D78. This "Na^+-stabilized" receptor is recognized by the agonist bradykinin with 100-fold weaker affinity [$K_i = (1 \pm 0.2) \times 10^{-7}$ M] compared to the "Na^+-free" B_2 receptor [$K_i = (1 \pm 0.1) \times 10^{-9}$ M]. Hence, similarly to other G protein-coupled receptors, the negative charge of the conserved aspartate in TM-II (D78) is responsible for the allosteric modulation of agonist binding by Na^+ ions. This finding together with the high sensitivity of B_2 agonist binding to the presence of Na^+ makes the B_2 receptor an appropriate model to study the effect of Na^+ on receptor activation.

Affinity of Na^+ to the B_2 Receptor. Prior to the analysis of the effect of Na^+ on B_2 receptor activation we determined the apparent affinity of Na^+ to the B_2 receptor to estimate the amount of "Na^+-stabilized" B_2 receptors under physiological Na^+ concentration. By determining the affinity of Na^+ to the receptor we assume that Na^+ ions directly bind to the receptor with the conserved aspartate in TM-II of 7TM receptors being at least part of a Na^+ binding site. Such a conclusion, i.e., that the basis of the Na^+ effect resides within the receptor, is strongly suggested by the fact that a purified hydrophobic core of the α_2-adrenergic receptor retains allosteric modulation of ligand binding by Na^+ (Wilson et al., 1990). Since it was not possible to determine the affinity of Na^+ to the B_2 receptor by competition binding with B_2 ligands due to localization of the respective binding sites on different parts of the receptor, we determined the number of Na^+-bound (Na^+-stabilized) B_2 receptors at a given Na^+ concentration indirectly: the number of Na^+-stabilized B_2 receptors was calculated from the difference between the total number of B_2 receptors (B_{max}) determined in "Na^+-free" buffer, and the number of "Na^+-free" receptors at a defined Na^+ concentration. This calculation was possible since the Na^+-stabilized B_2 receptors with an affinity for bradykinin of 1×10^{-7} M are not detected (less than 5%, cf. Figure 2) when the number of binding sites is determined by saturation binding with [^{3}H]bradykinin applied in a concentration range of 1×10^{-11} to 5×10^{-9} M. After Scatchard transformation of the binding data the K_D for the binding of Na^+ to the B_2 receptor was determined, it was 0.15 ± 0.01 M (Figure 3A, B). Thus at physiological intracellular Na^+ (38−40 mM; Motulsky & Insel, 1983) the fraction of Na^+-stabilized receptors with low affinity for bradykinin represents 20% of the total number of B_2 receptors.

Ion selectivity of the Na^+ Effect. We wanted to examine the biological effect of intracellular Na^+ on receptor activation. However, the majority of intracellular free ions are K^+ ions with a concentration of 157 mM. We therefore asked whether the effect of Na^+ ions on agonist binding is selective for Na^+ or whether other monovalent cations, e.g., K^+ ions, also influence agonist binding. We performed saturation binding and determined the affinity and the apparent number of binding sites for [^{3}H]bradykinin and for [^{3}H]Phe^{5}HOE140 (each applied in concentrations ranging between 1×10^{-10} and 1×10^{-8} M) in the presence of 138 mM of the monovalent cations Li^+, K^+, Rb^+, or Cs^+. On membranes of COS cells transfected with the rat B_2 receptor cDNA, the presence of 138 mM K^+, Rb^+ or Cs^+ had no effect on the affinity (not shown) and the number of binding sites for the B_2 agonist or antagonist (Figure 3C). Besides Na^+, only Li^+ ions decreased the apparent number of bradykinin binding sites (Figure 3C). Thus the effect of Na^+ ions on agonist binding is selective. Only ions with an ion radius of Na^+ or smaller decrease the apparent number of binding sites for bradykinin (Figure 3C). This selectivity may indicate that the monovalent ion binding site is within a pocket that excludes ions exceeding the size of Na^+.

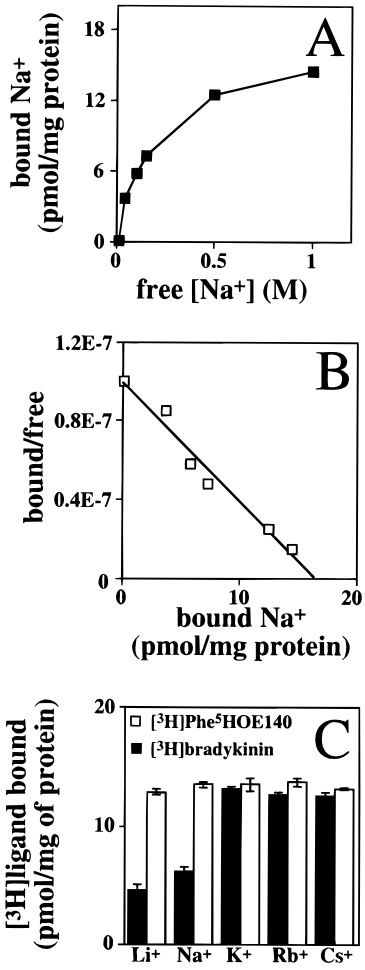


FIGURE 3: (A, B) Affinity of Na^+ to the B_2 receptor. (A) Number of Na^+-stabilized B_2 receptors was calculated from the difference between the apparent number of binding sites for [^{3}H]bradykinin determined at a concentration of 5×10^{-9} M in nominally Na^+-free buffer, and the apparent number of binding sites determined at a defined Na^+ concentration (10, 40, 100, 150, 500, and 1000 mM). Except for 1000 mM Na^+, the ion concentration of the buffers was maintained at 0.5 M by the addition of *N*-methyl-D-glucamine. (B) Scatchard transformation of the binding data from A. The K_D value for the binding of Na^+ to the B_2 receptor was calculated using the EBDA/Ligand program (Munson & Rodbard, 1980) and is the mean (± standard error) of three independent experiments. (C) Ion selectivity of agonist and antagonist binding. The apparent number of binding sites for [^{3}H]Phe^{5}HOE140 (□) and for [^{3}H]bradykinin (■) on membranes of COS cells transfected with rat B_2 receptor was determined in binding buffer where 138 mM *N*-methyl-D-glucamine had been exchanged for 138 mM Li^+, Na^+, K^+, Rb^+, or Cs^+. Data are representative of three independent experiments each performed in triplicate (± standard error).

Decrease of Intracellular Na^+ and Its Effect on Agonist-Independent B_2 Receptor Activation. Do intracellular Na^+ ions affect the B_2 receptor-mediated activation of a $G\alpha_q$-stimulated signal transduction cascade? In a first experiment we suppressed the interaction of Na^+ ions with the B_2 receptor by depleting the cells of intracellular Na^+. To this end we pre-incubated COS cells for 15 min in buffer where 138 mM NaCl had been replaced by 138 mM *N*-methyl-D-glucamine chloride prior to hormone stimulation. This treatment has been shown to decrease the Na^+ content of platelets 3-fold (Motulsky & Insel, 1983) and is expected to decrease the intracellular Na^+ content greatly in these or any cells due to the presence of Na^+/K^+-ATPases in all nucleated mammalian cells. Changes in the intracellular Na^+ content correlated with changes in the apparent number of binding sites for [^{3}H]bradykinin determined at 5×10^{-9} M with intact adherent cells. When the cells were incubated in physiological buffer with 148 mM Na^+ the apparent number of binding sites for [^{3}H]bradykinin were 80% of the total number of receptors determined with [^{3}H]Phe^{5}HOE140. In contrast, after preincubation of the cells in "low-Na^+" buffer with 10 mM Na^+ the apparent number of [^{3}H]bradykinin binding sites increased to 95%. According to Figure 3A,B, fractions of 20% or 5% of Na^+-stabilized B_2 receptors with low affinity for bradykinin correspond to intracellular Na^+ concentrations of 40 mM and 10 mM, respectively. These values coincide well with the intracellular Na^+ concentrations of platelets determined under similar conditions (Motulsky & Insel, 1983) indicating that preincubation of COS-1 cells in low-Na^+ buffer for 15 min efficiently decreased the intracellular Na^+ content of these cells.

We then determined the basal amount of inositolphosphates of COS cells transiently expressing wild-type rat B_2 receptors. The basal amount of inositolphosphates increased 2.8−3-fold after preincubation of the cells in low-Na^+ buffer (Figure 4A). Low-Na^+ buffer only affected cells expressing recombinant B_2 receptors and did not change the basal amount of inositolphosphates of mock-transfected COS cells (Figure 4A). This strongly suggests that the increase in basal inositolphosphates is mediated by activation of B_2 receptors. The increase in the basal amount of inositolphosphates was suppressed in the presence of U-73122 (Yule et al., 1992) an inhibitor of phospholipase(s) C (Figure 4A) indicating that the increase in the basal amount of inositolphosphates was due to activation of phospholipase(s)-C. To block Na^+/K^+-ATPases we incubated the cells with 30 μM ouabain. In the presence of ouabain, preincubation of the B_2 receptor expressing COS cells in low-Na^+ buffer did not lead to an increase in the basal amount of inositolphosphates as it did in the absence of ouabain. This confirms our previous notion that in low-Na^+ buffer the intracellular Na^+ is depleted via Na^+/K^+-ATPases and indicates that the Na^+ substitute, *N*-methyl-D-glucamine, does not stimulate B_2 receptors by itself. Together the preceding findings indicate that the decrease in intracellular Na^+ activates B_2 receptors in the absence of agonist. The activated receptors stimulate phospholipase(s)-C which induce the release of inositolphosphates.

Effect of Intracellular Na^+ on Agonist-Stimulated B_2 Receptor Activation. We investigated whether the decrease of intracellular Na^+ affects B_2 receptor activation stimulated by the agonist bradykinin. Stimulation with bradykinin of COS cells transiently expressing rat B_2 receptors induced the release of inositolphosphates with an $EC_{50} = (1.5 \pm 0.2) \times 10^{-9}$ M (Figure 4B). Compared to mock-transfected COS cells the expression of recombinant rat B_2 receptors (2.5−3 pmol/mg of protein) decreased the EC_{50} for the bradykinin-induced release of inositolphosphates and increased the maximum signal 3-fold.

Next we determined the potency and efficacy of bradykinin under decreased intracellular Na^+ content. When intracellular Na^+ was decreased by preincubation of the cells in low-Na^+ buffer, the intrinsic activity of bradykinin increased to 1.2 ± 0.05. The potency of bradykinin [$EC_{50} = (1.0 \pm 0.4) \times 10^{-9}$] M) was not significantly altered (Figure 4B).

We then asked whether we could reduce the activation of B_2 receptors by increasing the intracellular Na^+ content. To

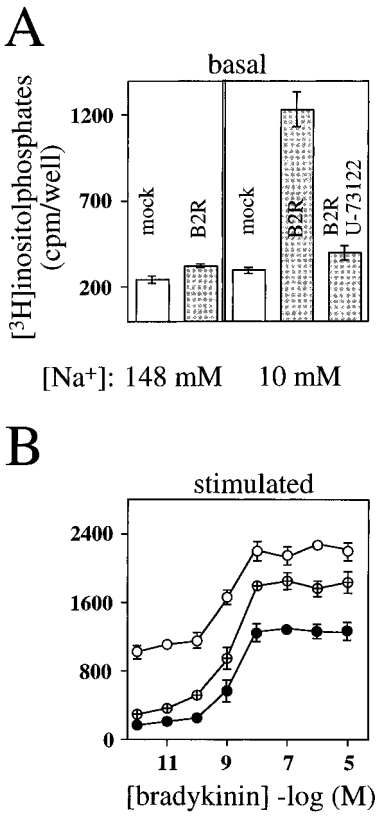


FIGURE 4: (A, B) Total inositolphosphates of COS-1 cells expressing wild-type rat B_2 receptor determined in the presence of different intracellular Na^+ concentrations. Mock-transfected COS-1 cells and cells expressing wild-type B_2 receptors were labeled with myo-[2-^{3}H]inositol. (A) Basal inositolphosphates of mock-transfected cells (□) or of COS cells expressing wild-type B_2 receptors (shaded bars) were determined. Cells were incubated for 15 min in buffer with 148 mM Na^+ or in buffer with 10 mM Na^+ with *N*-methyl-D-glucamine as Na^+ substitute. To block phospholipase(s)-C, 10 μM U-73122 was added to the cells 5 min prior to the experiment. Data are representative of four independent experiments each performed in triplicate (± standard error). (B) Dose−response curves for bradykinin-induced release in inositolphosphates on COS cells expressing wild-type rat B_2 receptor. Prior to the experiment cells were incubated for 15 min in incubation buffer with 148 mM Na^+ in the presence (●) or absence (⊕) of 30 μM monensin or in incubation buffer with 10 mM Na^+ (○). Bradykinin (1×10^{-12} M to 1×10^{-5} M) was then added to the cells for 15 min, and total inositolphosphates were determined. Experiments were performed in triplicate (± standard error), and EC_{50} values are the means (± standard error) of three independent experiments.

increase the intracellular Na^+ content we applied 30 μM of the Na^+ ionophore monensin previously reported to dissipate the Na^+ gradient of platelets (Motulsky & Insel, 1983), and we determined the bradykinin-stimulated release of inositolphosphates. The maximum release of inositolphosphates stimulated by bradykinin decreased by 28 ± 5% in the presence of monensin (Figure 4B). Thus the intrinsic activity of bradykinin decreased to 0.72 ± 0.05 under increased (148 mM) intracellular Na^+; the potency of bradykinin decreased slightly [$EC_{50} = (3 \pm 0.4) \times 10^{-9}$ M]. To exclude nonspecific effects of monensin on the B_2 signaling pathway we applied 30 μM monensin to cells in low-Na^+ buffer. The presence of monensin did not alter the dose−response curve of bradykinin in low-Na^+ buffer (not shown) indicating that besides equilibrating the extracellular with the intracellular Na^+ concentration, monensin had no additional effect on the activation of B_2 receptors.

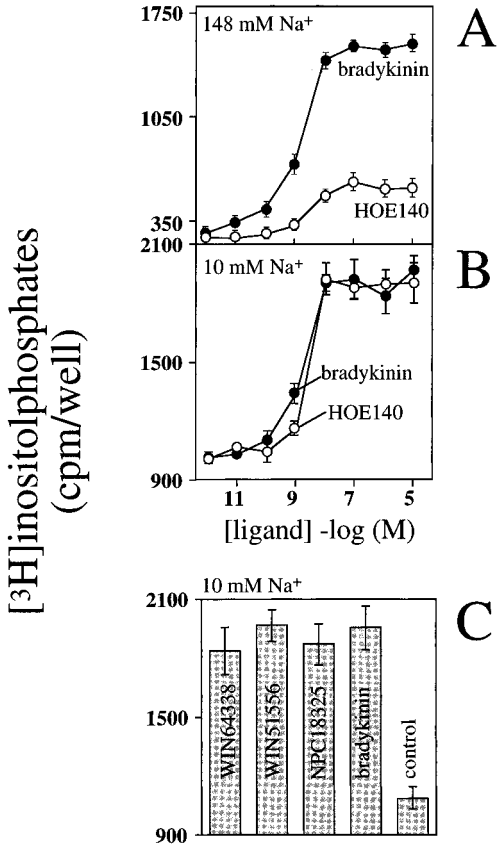


FIGURE 5: Agonistic activity of B_2 antagonists under decreased intracellular Na^+ (10 mM) on rat B_2 receptors expressed in COS cells. (A, B) Dose−response curve for the (●) bradykinin- and (○) HOE140-induced release in inositolphosphates determined in buffer with 148 mM (A) or 10 mM Na^+ (B). Data are the means (± standard error) of three independent experiments. (C) Release of inositolphosphates induced by B_2 antagonists (1 μM) or bradykinin determined in buffer with 10 mM Na^+. The indicated agent or buffer (control) was applied for 15 min. Data are representative of three independent experiments each performed in triplicate (± standard error).

Effect of Intracellular Na^+ Decrease on Potency and Intrinsic Activity of the B_2 Antagonist HOE140. We asked whether the activation of B_2 receptors by decreased intracellular Na^+ is blocked by the B_2 receptor antagonist HOE140. Unexpectedly, on rat B_2 receptors expressed in COS cells, HOE140 induced the release of inositolphosphates with an $EC_{50} = (6 \pm 0.1) \times 10^{-9}$ M (Figure 5A). The intrinsic activity (efficacy) of HOE140 compared to the full agonist bradykinin was 0.32 ± 0.02 (Figure 5A). This indicates that the B_2 specific antagonist HOE140 is a partial agonist on rat B_2 receptors expressed in COS cells. Partial agonistic activity of HOE140 has been previously reported for B_2 receptors in sheep (Félétou et al., 1994) where HOE140 displayed partial agonistic and full antagonistic properties depending on the organ tested.

Since HOE140 was a partial agonist on B_2 receptors expressed in COS cells we then analyzed whether the decrease in intracellular Na^+ altered the intrinsic activity of the partial agonist HOE140, and we preincubated the cells for 15 min in low-Na^+ buffer. Under low-Na^+ conditions the partial agonist HOE140 was apparently converted to a full agonist with an intrinsic activity identical with bradykinin (Figure 5B). Also the potency of HOE140 increased, the EC_{50} was $(6 \pm 0.1) \times 10^{-9}$ M in buffer with 148 mM Na^+

and $(2 \pm 0.1) \times 10^{-9}$ M under low-Na^+ conditions (Figure 5B).

To find out whether "novel" B_2 antagonists which in contrast to HOE140 are non-peptide antagonists or "pseudo-peptides" also display agonistic properties under low-Na^+ conditions, we applied WIN64338 and WIN51556 (Sawutz et al., 1994) as non-peptide antagonists and NPC18325 (Chakravarty et al., 1995) as a pseudo-peptide. All three antagonists tested were apparently converted to full agonists under low-Na^+ conditions (Figure 5C). As mentioned above COS-1 cells endogenously express minute amounts of B_2 receptors (cf. Figure 1A). In low-Na^+ buffer HOE140 also was a full agonist on the endogenously expressed B_2 receptors of COS cells (not shown). This observation indicates that the agonistic properties of the B_2 antagonist HOE140 on COS-1 cells may be due to yet undefined cellular components of COS-1 cells but not to altered B_2 receptor−effector coupling as a consequence of high amount of expressed B_2 receptors.

Expression of the D78N Rat B_2 Receptor Mutant in COS-1 Cells. To prevent the interaction of Na^+ ions with the B_2 receptor without affecting the Na^+ content of the cells, we removed the negatively charged aspartate D78 in TM-II and exchanged it for asparagine. This does not affect the affinity of agonist or antagonist (cf. Figures 1B and 2) but abolishes the interaction of Na^+ ions with the B_2 receptor (cf. Figure 2). The mutant receptor was transiently expressed in COS cells. Expression of the mutant receptor was verified by binding studies (Figure 1B, Figure 2) and immunoblotting analysis (Figure 6A). A mixture of antibodies to the different extracellular domains of the B_2 receptor (AbdAlla et al., 1996a) stained a protein of 55 ± 5 kDa in COS-1 cells expressing D78N-mutated (Figure 6A, lane 1) or wild-type (Figure 6A, lane 2) B_2 receptors, whereas this protein was absent in mock-transfected cells (Figure 6A, lane 3) indicating that the antibodies specifically cross-reacted with the expressed B_2 receptors. Intensities of the protein bands of the wild-type and the D78N-mutated B_2 receptor were similar, suggesting that equal amounts of receptors had been expressed and that the mutation of aspartate 78 to asparagine did not significantly alter the expression level of the receptor, a finding confirmed by saturation binding (Figure 2A).

Release of Inositolphosphates by the D78N Rat B_2 Receptor Mutant. Inositolphosphates of COS cells expressing D78N-mutated B_2 receptors were determined. Basal amounts of inositolphosphates were elevated 2.8−3-fold in cells expressing the mutant receptor compared to mock-transfected cells or to cells expressing wild-type B_2 receptor (Figure 6B). In this experiment COS-1 cells expressed a total amount of 2.6 pmol/mg of protein of wild-type B_2 receptors and 2.1 pmol/mg of protein of mutated receptors. The affinities of bradykinin to the mutant or to the wild-type B_2 receptor were identical (cf. Figure 1B). Thus the possibility that bradykinin was endogenously released by COS cells or that minute amounts of kinins in the growth medium lead to the observed increase in the basal amount of inositolphosphates of the D78N mutant expressing COS cells seems unlikely: these amounts of bradykinin would be equally potent in stimulating wild-type and mutant receptor. Therefore the finding that basal inositolphosphates of D78N-mutated B_2 receptor expressing cells were elevated 3-fold strongly suggests that the mutation of aspartate 78 to asparagine leads to (partial) constitutive activation of B_2 receptors. The EC_{50} values for

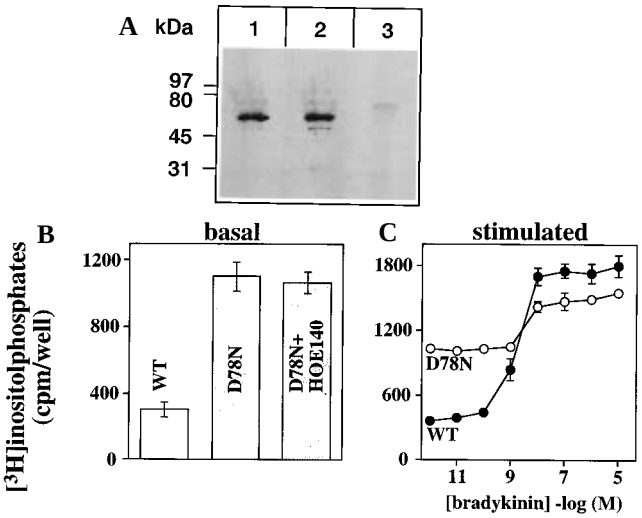


FIGURE 6: (A) Identification of wild-type and D78N-mutated B_2 receptors in immunoblots. Immunoblots of solubilized membranes of COS-1 cells expressing D78N-mutated (lane 1) or wild-type (lane 2) B_2 receptors were probed by a mixture of anti-peptide antisera to the different extracellular domains of the rat B_2 receptor. Antiserum dilution was 1:1000. Solubilized membranes from mock-transfected COS cells were included as a negative control (lane 3). (B) Basal release of inositolphosphates of D78N-mutated B_2 receptors. Basal release of inositolphosphates of COS cells expressing D78N-mutated or wild-type (WT) B_2 receptors was determined 15 min after the addition of buffer or of 5 μM HOE140. Cells were kept in incubation buffer with 148 mM Na^+. Data are representative of five independent experiments each performed in triplicate (± standard error). (C) Dose−response relationship of the bradykinin-induced release of inositolphosphates determined on COS cells expressing wild-type (●) or D78N-mutated (○) B_2 receptors. Experiments were performed in triplicate (± standard error) and EC_{50} values are the means (± standard error), of three independent experiments.

the bradykinin-stimulated increase in inositolphosphates were 1.5 ± 0.1 nM for the wild-type B_2 receptor and 4 ± 0.7 nM for the mutated receptor (Figure 6C); the intrinsic activity of bradykinin on the D78N mutant was reduced to 0.8 ± 0.05 compared to the wild-type B_2 receptor when identical amounts of B_2 receptors were expressed (Figure 6C). In contrast to the wild-type B_2 receptor, the intrinsic activity of bradykinin on the D78N mutant and basal inositolphosphate values were identical in buffer with 148 mM Na^+ and in low-Na^+ buffer with 10 mM Na^+ (not shown). Also, the presence of ouabain did not change the basal or the bradykinin-stimulated amount of inositolphosphates on COS cells expressing the D78N mutant (not shown). These findings demonstrate that the mutation abolished the effect of Na^+ ions on B_2 receptor activation. The B_2 antagonist HOE140 had no effect on the basal amount of inositolphosphates of the D78N mutant, and it was no partial agonist on this B_2 receptor mutant (Figure 6B).

A B_2 receptor mutant that does not undergo a conformational change in the presence of Na^+ ions is constitutively-activated in the absence of agonist. In contrast to constitutively-activated α_1- and β_2-adrenergic receptor mutants (Cotecchia et al., 1990; Samama et al., 1993) where agonists have increased potency, the potency and efficacy of bradykinin on the constitutively activated B_2 receptor mutant were decreased, and the partial agonist HOE140 was converted to a full antagonist. This may be due to increased desensitization of the signaling pathway as has been suggested for a constitutive active opsin mutant (Li et al., 1995). A

similar phenomenon, i.e., that a constitutive active receptor mutant was no longer activated by agonists or was activated with decreased potency of the agonist has been previously observed for mutants of the melanocyte-stimulating hormone receptor (Robbins et al., 1993).

Effect of Na^+ Ions on the Activation of Human B_2 Receptors in a Native Cell System. So far, the effect of Na^+ ions on B_2 receptor activation was investigated on recombinant rat B_2 receptors expressed in COS-1 cells. We therefore asked whether decrease of intracellular Na^+ also affected B_2 receptors of a native cell system, and we chose human foreskin fibroblasts (HF-15) endogenously expressing functional B_2 receptors (0.5−1 pmol B_2 receptors/mg of protein). Expression of human B_2 receptors was verified by binding studies with [^{3}H]bradykinin, and by photoaffinity labeling of the receptors with the photoreactive B_2 antagonist BBA-Lys^{0}HOE140 and detection of BBA-Lys^{0}HOE140-labeled B_2 receptors in immunoblots by anti-HOE140 antibodies (Figure 7A). This ligand, which binds to B_2 receptors with an affinity of $K_i = (1.5 \pm 0.3) \times 10^{-9}$ M, specifically labeled B_2 receptors. The molecular mass of 69 ± 3 kDa (Figure 7A, lanes 1and 2) is in good agreement with previous findings (AbdAlla et al., 1993). The labeling of the B_2 receptor was suppressed when the cross-linking was performed in the presence of a 1000-fold molar excess of bradykinin (Figure 7A, lane 3). This finding further supports that BBA-Lys^{0}HOE140 specifically labeled B_2 receptors of HF-15 cells.

We determined the basal amount of inositolphosphates of HF-15 cells. When the cells were pre-incubated for 15 min in low-Na^+ buffer, the basal amount of inositolphosphates of HF-15 cells increased 2.5-fold compared to cells incubated for 15 min in physiological buffer with 148 mM Na^+. Hence, a decrease in intracellular Na^+ also activates human B_2 receptors endogenously expressed on primary cells. This observation coincides with our previous findings made with recombinant B_2 receptors expressed in COS cells. The basal increase in inositolphosphates of HF-15 cells was blocked by 5 μM of the B_2 antagonist HOE140, indicating that the increase in basal inositolphosphates was mediated by activation of B_2 receptors (Figure 7B).

In HF-15 cells unlike COS-1 cells, HOE140 seems to act as an inverse agonist but not as a partial agonist. Inverse agonism of HOE140 has been reported previously for B_2 receptors in rat myometrial cells (Leeb-Lundberg et al., 1994). The observation that HOE140 behaves as a partial agonist on rat B_2 receptors expressed in COS cells and as an inverse agonist on rat B_2 receptors of myometrial cells may be a consequence of using transient expression in COS cells versus native B_2 receptor expression in myometrial cells. However, since HOE140 is a partial agonist on B_2 receptors endogenously expressed in COS cells (see above), the differential agonistic/antagonistic properties of HOE140 may also reflect that the equilibrium of the active and inactive B_2 receptor conformations—according to the ternary complex model of receptor activation (Samama et al., 1993)—may be different in different cell systems.

Whole Cell Phosphorylation of B_2 Receptors Under Low Na^+ Conditions. Phosphorylation of 7TM receptors is a consequence of receptor activation (Ren et al., 1993). We therefore asked whether the increase in basal activity of the B_2 receptor under low-Na^+ conditions is accompanied by an increased phosphorylation of the B_2 receptor. We

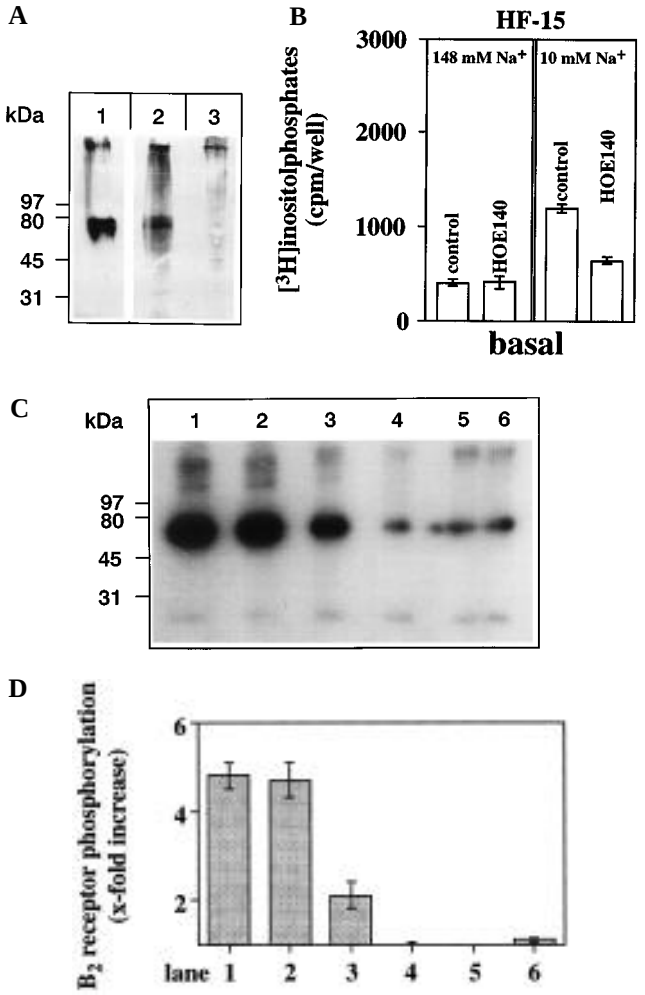


FIGURE 7: (A) Identification of BBA-Lys^{0}HOE140-labeled B_2 receptors of HF-15 cells in immunoblotting. To identify human B2 receptors of HF-15 cells we performed cross-linking of BBA-Lys^{0}HOE140 to the B_2 receptor and identified the BBA-Lys^{0}HOE140-labeled B_2 receptors in immunoblots probed by polyclonal (lane 1) or monoclonal (lanes 2 and 3) anti-HOE140 antibodies. To control the specificity of the cross-linking we performed the reaction in the absence (lane 2) or presence of a 1000-fold molar excess of bradykinin (lane 3). (B) Basal release of inositolphosphates of HF-15 cells under low-Na^+ conditions. The experiment with HF-15 cells was similarly performed as with COS cells. HF-15 cells were incubated for 15 min in buffer with 148 mM Na^+ or in buffer with 10 mM Na^+, and basal inositolphosphates were determined. To suppress basal activation of B_2 receptors, HOE140 (5 μM) was added 5 min prior to buffer exchange into low-Na^+ buffer with 10 mM Na^+ or into buffer with 148 mM Na^+, and HOE140 was present throughout the experiment. Data are representative of three independent experiments each performed in triplicate (± standard error). (C) Whole cell phosphorylation of HF-15 cells. The [^{32}P]-labeled HF-15 cells were incubated for 10 min in incubation buffer with 148 mM Na^+ (lanes 1, 5, and 6) or in buffer with 10 mM Na^+ (lanes 2−4), and then 500 nM bradykinin (lanes 1 and 2) or 5 μM HOE140 followed by 500 nM bradykinin (lane 5) was added for 5 min to the cells. Basal phosphorylation was determined on cells kept in incubation buffer with 10 mM Na^+ (lane 3) or kept in buffer with 148 mM Na^+ (lane 6). To suppress basal activation of B_2 receptors, HOE140 (5 μM) was added 5 min prior to buffer exchange into low-Na^+ buffer, and HOE140 was present throughout the experiment (lane 4). The [^{32}P]-labeled B_2 receptors were immunoprecipitated with a mixture of antibodies raised against peptides from the carboxy terminal and the different extracellular domains of the B_2 receptor, separated by SDS−PAGE, and visualized by autoradiography. (D) After SDS−PAGE, the bands corresponding to the region of the B_2 receptor were cut and counted; the *n*-fold increase over basal (determined in buffer with 148 mM Na^+) was calculated. Data shown here are the means ± standard error from three separate experiments.

immunoprecipitated B_2 receptors from [^{32}P]-labeled HF-15 cells. For immunoprecipitation we used a mixture of antibodies to peptides derived from the carboxy terminal and the different extracellular domains of the human B_2 receptor (AbdAlla et al., 1996a). The antibodies cross-react with denatured and native B_2 receptors in immunoprinting (Figure 6A), immunoaffinity chromatography, and immunofluorescence (AbdAlla et al., 1996a) and have been applied to enrich B_2 receptors of HF-15 cells for amino-terminal protein sequencing (AbdAlla et al., 1996b). Stimulation of HF-15 cells for 5 min by 500 nM bradykinin resulted in a 4.8-fold increase in the phosphorylation of the B_2 receptor protein (Figure 7C,D, lane 1) compared to non-stimulated control cells (Figure 7C,D, lane 6). This indicates that analogous to several other 7TM receptors, B_2 receptors undergo agonist-induced phosphorylation. This agonist-induced phosphorylation was suppressed in the presence of 5 μM of the B_2 antagonist HOE140 (Figure 7C,D, lane 5), indicating that phosphorylation of the B_2 receptor protein was mediated specifically by activation of B_2 receptors.

Next we analyzed the phosphorylation of B_2 receptors under decreased intracellular Na^+. The bradykinin-stimulated phosphorylation of the B_2 receptor was not significantly altered by preincubation of the cells in low-Na^+ buffer (Figure 7C,D, lane 2). In contrast, basal phosphorylation of the B_2 receptor in the absence of agonist increased 1.9-fold (Figure 7C,D, lane 3) when the cells were preincubated for 10 min in low Na^+ buffer with 10 mM Na^+. This basal increase in phosphorylation was suppressed in the presence of 5 μM of the B_2 antagonist HOE140 (Figure 7C,D, lane 4), indicating that the increase in basal phosphorylation of the B_2 receptor was mediated by B_2 receptor activation. This finding coincides with the preceding experiment where we detected an increase in the basal release of inositolphosphates under decreased intracellular Na^+, and it supports the concept that a decrease of the intracellular Na^+ content activates B_2 receptors in the absence of agonist.

DISCUSSION

Seven transmembrane domain (7TM) receptors are supposed to exist in equilibrium between an inactive R and active R* conformation (Samama et al., 1993; Bond et al., 1995). We investigated the effect of Na^+ on ligand binding and activation of the B_2 receptor. The presence of Na^+ decreased the affinity of B_2 agonists up to 100-fold and dose-dependently suppressed B_2 receptor activation. Within the ternary complex model of receptor activation (Samama et al., 1993) the Na^+-induced or -stabilized B_2 receptor conformation with reduced affinity for agonists (100-fold for bradykinin, 2-fold for the partial agonist HOE140) ressembles the inactive R state. This finding may reflect that the B_2 receptor exists in equilibrium between at least two different receptor conformations: an active state R* which is readily able to couple to G proteins and favored by agonists, and an inactive state R which is not readily able to couple to G proteins and which is favored by Na^+. The existence of different B_2 receptor states is in agreement with previous findings made with native B_2 receptors of rat myometrial cells (Leeb-Lundberg et al., 1994). We therefore hypothesize that Na^+ ions dose-dependently shift the B_2 receptor equilibrium of R and R* toward R by stabilizing or inducing an inactive R-like B_2 receptor state.

This hypothesis based on binding data was further supported by experiments on the effect of Na^+ on B_2 receptor activation. Rat B_2 receptors were transiently expressed in COS-1 cells to study the effect of intracellular Na^+ on receptor activation. Decrease of intracellular Na^+ from 40 to 10 mM partially activated B_2 receptors in the absence of agonist, increased the efficacy of the agonist bradykinin from 1.0 to 1.2, and apparently converted the partial agonist HOE140 to a full agonist. In contrast, increase of intracellular Na^+ from 40 to 148 mM decreased the efficacy of bradykinin to 0.72. In addition to experiments where we changed the intracellular Na^+ content we prevented the interaction of Na^+ with the B_2 receptor in a B_2 receptor mutant. The exchange of a conserved aspartate in TM-II for asparagine which abolished the effect of Na^+ on agonist binding and receptor activation, constitutively activated the B_2 receptor in the absence of agonist. Thus within the ternary complex model of receptor activation we explain our data to mean that Na^+ ions binding to the B_2 receptor stabilize or induce an inactive R-like state of the B_2 receptor, thereby shifting the equilibrium of the inactive R and active R* state toward the inactive R state. Decreasing the intracellular Na^+ seems to increase the fraction of receptors in the active R*-like state and to decrease those in the inactive R-like state. These changes in the equilibrium of R and R* may account for the observed Na^+-dependent changes in B_2 receptor activation.

The agonist-dependent alteration of the balance between receptor activation and inactivation is the basis of receptor function. Disregulation of the balance—constitutive receptor activity—leads to diseases (Lefkowitz, 1993). Though for several 7TM receptors, mutants have been identified which shift the equilibrium toward the active R* conformation (Lefkowitz et al., 1993), for visual opsin the control mechanism of receptor activation is the best understood (Robinson et al., 1992). The chromophore 11-cis retinal covalently attached to the ϵ-amino group of lysine^{296} in rhodopsin stabilizes an inactive receptor conformation thereby preventing receptor activation in the absence of agonist (light). In addition to the mechanism for rhodopsin, our data suggest that the physiological intracellular Na^+ concentration (40 mM) is sufficent to suppress agonist-independent activation of B_2 receptors endogenously expressed on primary human fibroblast cells. This was evident when the intracelluar Na^+ was decreased from 40 to 10 mM. Decrease of intracellular Na^+ activated B_2 receptors in the absence of agonist as demonstrated by a 2.5-fold increase in the basal release of inositolphosphates and by a 1.9-fold increase in the basal phosphorylation of the B_2 receptor protein. Hence, binding of Na^+ ions to the B_2 receptor may provide a mechanism to suppress agonist-independent activation of the B_2 receptor *in vivo*.

The antagonistic action of divalent metal ions on a 7TM receptor mutant has been previously reported for the NK-1 receptor where an antagonist binding site had been converted to a metal ion binding site (Elling et al., 1995). Aspartate 78 in TM-II of the B_2 receptor seems to be an essential part of an endogenous Na^+ ion binding site. Analogous to the antagonistic action of Zn^{2+} ions on the NK-1 receptor mutant (Elling et al., 1995), Na^+ ions seem to stabilize an inactive receptor conformation of the B_2 receptor by binding to D78. This aspartate is part of the LAXAD motif present in most 7TM receptors (Baldwin, 1993). For many systems studied

removal of the negative charge in TM-II results in a parallel loss of anterograde (agonist−receptor−G protein function) and retrograde (G protein−receptor−agonist affinity) communication between receptor and G protein (Ceresa & Limbird, 1994). Though these data apparently contrast with the observations made with the analogous B_2 receptor mutant D78N which is constitutively activated, all of these observations commonly reflect the importance of this residue in the control of receptor activation. It will be interesting to learn whether the mechanism found for the B_2 receptor, i.e., that the Na^+ ion binding site is a regulator of agonist-independent receptor activation is true for 7TM receptors other than the B_2 receptor. Taking into account that, e.g., elevated cellular Na^+ is a key mediator of hypertension, then stabilization and/or induction of an inactive B_2 receptor conformation by intracellular Na^+ ions may be involved in the pathogenesis of hypertension by decreasing the vasodilatory action of B_2 receptors. Future work will be necessary to understand the full impact of changes in cellular Na^+ on (patho)physiological processes.

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REFERENCES

AbdAlla, S., Buschko, J., Quitterer, U., Maidhof, A., Haasemann, M., Breipohl, G., Knolle, J., & Müller-Esterl, W. (1993) *J. Biol. Chem. 268*, 17277−17285.

AbdAlla, S., Quitterer, U., Grigoriev, S., Maidhof, A., Haasemann, M., Jarnagin, K., & Müller-Esterl, W. (1996a) *J. Biol. Chem. 271*, 1748−1755.

AbdAlla, S., Godovac-Zimmermann, J., Braun, A., Roscher, A. A., Müller-Esterl, W., & Quitterer, U. (1996b) *Biochemistry 35*, 7514−7519.

Baldwin, J. M. (1993) *EMBO J. 12*, 1693−1703.

Bond, R. A., Leff, P., Johnson, T. D., Milano, C. A., Rockman, H. A., McMinn, T. R., Apparsundaram, S., Hyek, M. F., Kenakin, T. P., Allen, L. F., & Lefkowitz R., J. (1995) *Nature 374*, 272−276.

Ceresa, B. P., & Limbird, L. E. (1994) *J. Biol. Chem. 269*, 29557−29564.

Chakravarty, S., Connolly, M., & Kyle, D. J. (1995) *Pept. Res. 8*, 16−19.

Cotecchia, S., Exum, S., Caron, M. G., Lefkowitz, R. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2896−2900.

Elling, C. E., Nielsen, S. M., & Schwartz, T. W. (1995) *Nature 374*, 74−77.

Félétou, M., Germain, M., Thurieau, C., Fauchère, J.-L., & Canet, E. (1994) *Br. J. Pharmacol. 112*, 683−689.

Freedman, R., & Jarnagin, K., (1992) in *Recent Progress on Kinins* (Fritz, H., Müller-Esterl, W., Jochum, M., Roscher, A. A., & Luppertz, K., Eds.) pp 487−496, Birkhäuser, Basel.

Hess, J. F., Borkowski, J. A., Young, G. S., Strader, C. D., & Ransom, R. W. (1992) *Biochem. Biophys. Res. Commun. 184*, 260−268.

Horstman, D. A., Brandon, S., Wilson, A. L., Guyer, C. A., Cragoe, E. J., & Limbird, L. (1990) *J. Biol. Chem. 265*, 21590−21595.

Jones, S., Brown, D. A., Milligan, G., Willer, E., Buckley, N. J., & Caulfield, M. P. (1995) *Neuron 14*, 399−405.

Kyhse-Anderson, J. (1984) *J. Biochem. Biophys. Methods 10*, 203−209.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Leeb-Lundberg, L. M., Mathis, S. A., & Herzig, M. C. S. (1994) *J. Biol. Chem. 269*, 25970−25973.

Lefkowitz, R. J. (1993) *Nature 365*, 603−604.

Lefkowitz, R. J., Cotecchia, S., Samama, P., & Costa, T. (1993) *Trends Pharmacol. Sci. 14*, 303−307.

Li, T., Franson, W. K., Gordon, J. W., Berson, E. L., & Dryja, T. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3551−3555.

McCann, J. D., Bhalla, R. C., & Welsh, M. J. (1989) *Am. J. Physiol. 257*, L116−L124.

McEachern, A. E., Shelton, E., Bhakta, S., Obernolte, R., Bach, C., Zuppan, P., Fujisaki, J., Aldrich, R. W., & Jarnagin, K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7724−7728.

Motulsky, H. J.& Insel, P. A. (1983) *J. Biol. Chem. 258*, 3913−3919.

Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220−239.

Neve, K. A., Cox, B. A., Hinningsen, R. A., Spanoyannis, A., & Neve, R. L. (1993) *Mol. Pharmacol. 39*, 733−737.

Pert, C. B.& Snyder, S. (1974) *Mol. Pharmacol. 10*, 868−879.

Quitterer, U., Schroeder, C., Müller-Esterl, W., & Rehm, H. (1995) *J. Biol. Chem. 270*, 1992−1999.

Regoli, D., & Barabé, J. (1988) *Methods Enzymol. 163*, 210−230.

Ren, Q., Kurose, H., Lefkowitz, R. J., & Cotecchia, S. (1993) *J. Biol. Chem. 268*, 16483−16487.

Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K. G., & Cone, R. D. (1993) *Cell 72*, 827−834.

Robinson, P. R., Cohen, G. B., Zhukovsky, E., & Oprian, D. D. (1992) *Neuron 9*, 719−725.

Roscher, A. A., Manganiello, V. C., Jelsema, C. L., & Moss, J. (1983) *J. Clin. Invest. 72*, 626−635.

Samama, P., Cotecchia, S., Costa, T., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 4625−36.

Sawutz, D. G., Salvino, J. M., Dolle, R. E., Casiano, F., Ward, S. J., Faunce, D. M., Douty, B. D., Baizman, E., Awad, M. M., et al (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4693−4697.

Takebe, Y., Seiki, M., Fujisawa, J.-I., Hoy, P., Yokota, K., Arai, K.-I., Yoshida, M., & Arai, N. (1988) *Mol. Cell. Biol. 8*, 466−472.

Tsai, B. S., & Lefkowitz, R. J. (1978) *Mol. Pharmacol 14*, 540−548.

Wilson, A. L., Guyer, C. A., Cragoe, E. J., & Limbird, L. E. (1990) *J. Biol. Chem. 265*, 17318−17322.

Wirth, K., Hock, F. J., Albus, U., Linz, W., Alpermann, H. G., Anagnostopoulos, H., Henke, S., Breipohl, G., König, W., Knolle, J., & Schölkens, B. A. (1991) *Br. J. Pharmacol. 102*, 774−777.

Yule, D. I., & Williams, J. A. (1992) *J. Biol. Chem. 267*, 13830−13835.

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