Specific Receptor–Guanine Nucleotide Binding Protein Interaction Mediates the Release of Endothelium-Derived Relaxing Factor

James K. Liao and Charles J. Homcy

High affinity agonist-binding (HAB) sites are formed from specific receptor interaction with guanine nucleotide–binding (G) proteins. To determine whether the release of endothelium-derived relaxing factor (EDRF) is regulated by specific receptor–G protein coupling, we treated bovine aortic endothelial cells with 100 ng/ml pertussis toxin (PTX) for 16 hours to effect receptor–G protein uncoupling. The degree of receptor uncoupling as measured by the loss of HAB sites for the α2-adrenergic receptor and bradykinin receptor was assessed by radioligand binding studies using partially purified bovine aortic endothelial cell membranes. The release of EDRF in response to UK14304 (an α2-adrenergic receptor agonist) and bradykinin stimulation was measured with a bioassay apparatus. The G protein isofoms were characterized by Western blotting, and complete ADP-ribosylation of these proteins was confirmed by PTX-catalyzed [32P]NAD ribosylation. PTX produced a greater inhibition of EDRF release via the α2-adrenergic receptor pathway compared with the bradykinin receptor pathway (80% versus 46%, p<0.01). This corresponded to the loss of HAB sites from the α2-adrenergic receptor and bradykinin receptor pathway (72% versus 46%, p<0.01) as compared with complete loss of both HAB sites in the presence of GppNHp (0.1 mM). Since loss of HAB sites from PTX-mediated receptor uncoupling parallels the inhibition of EDRF release, these data suggest that G proteins contribute to a greater proportion of HAB sites derived from α2-adrenergic receptor rather than bradykinin receptor interaction and that the inhibition of EDRF release by PTX is mainly due to the loss of these HAB sites. The degree of HAB site formation from specific receptor–G protein coupling may serve as one mechanism for regulating EDRF release via different cell surface receptors. (Circulation Research 1992;70:1018–1026)

KEY WORDS • α2-adrenergic receptor • bradykinin receptor • radioligand binding studies • high affinity agonist-binding sites • pertussis toxin

Stimulation of diverse membrane receptors on endothelial cells causes the release of a potent vasodilator, endothelium-derived relaxing factor (EDRF). However, little is known concerning how these various receptor signals are transduced across cellular membranes. Recent evidence suggests that guanine nucleotide–binding (G) proteins may be important in coupling certain of these receptors to EDRF release. In porcine coronary arteries, endothelium-dependent relaxations to UK14304, an α2-adrenergic receptor agonist, are almost completely inhibited by pertussis toxin (PTX), whereas similar relaxations to bradykinin (BK) and the calcium ionophore A23187 are relatively unaffected. Further studies have shown that persistent endothelial dysfunction (i.e., EDRF release) in regenerating porcine coronary arterial endothelium may, in part, be due to abnormalities in receptor–G protein coupling. None of these studies, however, showed that PTX actually caused receptor–G protein uncoupling via ADP-ribosylation of the Gs subunit. Thus, the role of Gs proteins in mediating EDRF release has been implicated but not clearly demonstrated.

One method of assessing receptor–G protein coupling is by determining the presence of high affinity agonist-binding (HAB) sites. These sites are produced as a result of receptor–G protein interaction. Such interaction produces a “higher affinity receptor” that is capable of binding agonists with a much lower Kd. This productive interaction between receptor and G protein thereby facilitates the triggering of physiological responses upon hormone binding. The purpose of this study, therefore, is to characterize the components of two different receptor-mediated EDRF release pathways and to show that the formation of HAB sites, as a measure of receptor–G protein coupling, is differentially involved in EDRF release.

Materials and Methods

All tissue culture reagents, unless otherwise stated, were obtained from JRH Bioscience. Rabbit anti-factor
VIII immunoglobulin was obtained from Accurate Chemical and Scientific Corp., Westbury, N.Y. PTX, indomethacin, phenylmethylsulfonyl fluoride, protaglandin F₂α, (--)-epinephrine, (--)-propranolol, t-phenylephrine, ascorbic acid, calcium ionophore A23187, BK, des-Arg³[Leu⁴]BK, and [Thi²,D-Phe⁶]-BK were purchased from Sigma Chemical Co., St. Louis, Mo. Prazosin, an α₁-adrenergic antagonist, and UK14304, an α₂-adrenergic agonist, were gifts from Pfizer, Groton, Conn., and Kent, U.K. [³²P]UK14304 (62.7 Ci/mmol), [³²P]prazosin (77.8 Ci/mmol), [³²P]rauwolscine (76.7 Ci/mmol), [³²P]BK (121.6 Ci/mmol), and [³²P]NAD (30 Ci/mmol) were supplied by New England Nuclear, Boston. Rauwolscine was obtained from Roth, FRG. Oxymetazoline was obtained from Schering Corp., Bloomfield, N.J. Idazoxan was obtained from Reckitt-Colman, Kingston Upon Hull, UK. The polyclonal rabbit antiserum P₃ and P₄ and rabbit anti-endothelin-1 immunoglobulin were raised in our laboratory. The EC/2 and SW/1 antiserum were purchased from Du Pont Pharmaceuticals, Wilmington, Del. The Western blotting kit (Enhanced ChemiLuminescence) using horseradish peroxidase and luminol was obtained from Amersham Corp., Arlington Heights, Ill. Immobilon-P was supplied by Millipore Corp., Bedford, Mass. Recombinant α₂ and α₃ were generously provided by Lutz Birnbaumer and Juan Codina, Baylor, Houston, Tex.

Cell Culture

Fresh bovine aortas were obtained at a local slaughterhouse. Bovine aortic endothelial cells (BAECs) were harvested by gentle scraping and placed in a growth medium containing Dulbecco’s modified Eagle’s medium supplemented with 5 mM t-glutamine (GIBCO Laboratories, Grand Island, N.Y.), 10% fetal calf serum (HyClone Laboratories Inc., Logan, Utah), and an antibiotic mixture of penicillin (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (250 ng/ml). The cell culture was incubated at 37°C with 5% CO₂–95% air. A relatively pure BAEC culture (<5% fibroblasts and smooth muscle cells) was obtained after three cell passages and confirmed by a Nomarski optical microscope (×40 objective, model IC M 405, Zeiss), which showed contact inhibition and typical morphological features of endothelial cells (i.e., cobblestone, polygonal appearance) and by immunofluorescence staining using rabbit anti-factor VIII and anti-endothelin antibodies, followed by horseradish peroxidase–labeled goat anti-rabbit immunoglobulin and a monoethylcarbazole. For the bioassy of EDRF release, the BAECs were scraped from 150x15-mm gelatin-coated culture dishes and transferred onto 1.5 g (6x10⁶ beads) of hydrated microcarrier beads coated with denatured collagen (CytoDex 3, Pharmacia) and grown in suspension using a microcarrier culture vessel and stirring system (Bellco Glass). The suspension of cells and beads was initially placed in 100 ml media and stirred at 15 rpm for 2 minutes every 30 minutes for the first 4 hours to allow for optimal seeding of cells onto the beads. The system was then stirred continuously at 20 rpm with 250 ml media until the cells were confluent on the beads (~80 cells per bead), which usually occurred 3–4 days after seeding. All passages were performed with a disposable cell scraper (Costar Corp., Cambridge, Mass.), and only endothelial cells of less than six passages were used. The BAECs were then treated with PTX (100 ng/ml) for 16 hours before any experiment.

Membrane Purification

The BAECs were suspended in ice-cold lysis buffer (0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 5 mM Tris-HCl, pH 7.4), disrupted by a Dounce homogenizer (10 strokes twice), and centrifuged at 40,000g for 30 minutes at 4°C. The crude pellet was washed twice with ice-cold suspension buffer (100 mM Tris- HCl, 5 mM MgCl₂, 0.6 mM EDTA, pH 7.4) and then centrifuged at 3,000g for 10 minutes. The pellet was discarded, and the supernatant was centrifuged again at 40,000g for 45 minutes at 4°C. The resulting pellet containing the enriched membrane preparation was then resuspended at a protein concentration of 2–2.5 mg/ml as determined by the method of Lowry et al.9

Characterization of Receptors on Endothelial Cells

Characterization of the α₁-adrenergic receptor was performed with the agonist [³²P]UK14304 (5 nM) in the presence and absence of prazosin (10 μM), rauwolscine (1 μM), or oxymetazoline (1 μM). Nonspecific binding was determined in the presence of (–)-epinephrine (0.1 mM). The reaction mixture (100 μg membrane in a total volume of 100 μl) was allowed to equilibrate at room temperature (23°C) with gentle shaking for 45 minutes. The assays were terminated by rapid filtration on GF/C filters (Whatman Inc., Clifton, N.J.). Each filter was counted for 2 minutes in a liquid scintillation counter (model LS 1800, Beckman Instruments Inc., Fullerton, Calif.). The binding studies for the BK receptor were performed in a similar fashion with the following exceptions: The buffer, in addition, contained bacitracin (140 μg/ml), captopril (1 μM, E.R. Squibb & Sons, Inc., Princeton, N.J.), dithiothreitol (1 mM), 1,10-phenanthroline (1 mM), and bovine serum albumin (0.1%). All reaction tubes and filters were pretreated overnight with 0.1% bovine serum albumin and 0.1% polyethyleneimine, respectively. Binding of the radioligand, [³²P]BK (5 nM) was competed with 10 μM des-Arg³[Leu⁴]BK (a BK, antagonist) or 10 μM [Thi²,D-Phe⁶]-BK (a BK₂ antagonist) at 4°C for 90 minutes. Nonspecific binding was determined in the presence of BK (1 μM).

EDRF Bioassay

The EDRF bioassay apparatus consisted of an open-perfusion system, a mounted ring assembly attached to a force transducer for measuring vascular tension, and bioassay tissues. The system was constantly perfused at 4 ml/min by a roller pump (Minipuls 2, Gilson) with modified Krebs-Ringer bicarbonate solution of the following concentrations (mM): NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, EDTA 0.026, and glucose 11.1. The solution was aerated with 95% O₂–5% CO₂ and kept at 37°C by means of a thermocirculator (Lauda MS, Brinkmann Instruments, Inc., Westbury, N.Y.).

Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 200–250 g were killed by decapitation. After removal of the aortas, the adherent loose connective and adipose
tissues were dissected away. The endothelium was removed by gently rubbing the lumen with a dry cotton swab. The denuded aorta was then cut into 4-mm cylindrical strips and mounted on a bioassay ring connected to an isometric force transducer (model FT03C, Grass Instrument Co., Quincy, Mass.). Changes in isometric force resulting from relaxation or contraction of the bioassay tissue were recorded on a polygraph (model BD 41, Kipp & Zonen, Bohemia, N.Y.). In addition to the main perfusion port (4 ml/min), the denuded aorta was also directly perfused with Krebs’ solution through a second port at 2 ml/min. The total perfusion reaching the smooth muscle bioassay tissue was 6 ml/min. In a stepwise manner, the aorta was passively stretched until maximum contractions were obtained with repeated stimulation by KCl (80 mM). Preliminary studies showed that 1 g of tension was the optimal passive tension for isolated rat aortic rings.

To eliminate the effects of prostanoids, we added indomethacin (10 μM) to the perfusate, and the stretched artery was allowed to equilibrate for 60 minutes. Active tension (70% maximal contraction, 1.8-2 g) was produced by the addition of L-phenylephrine (5 μM) with ascorbic acid (0.1 mM) or, in some cases, prostanandin F2o (4 μM) to the second infusion port. The bioassay ring was then allowed to reach steady state (45 minutes). Before all experiments, both calcium ionophore A23187 and the agonist of interest were infused through the second port to determine whether these agents alone could directly elicit smooth muscle relaxation. Any bioassay tissue that relaxed in response to these agents was discarded. Several samples of denuded aorta were also examined by light and electron microscopy to assess the degree of endothelial removal.

Approximately 6×10^6 endothelial cells confluent on microcarrier beads were pretreated with PTX or buffer before being packed into a 70-μm chromatographic filter column (Evergreen Scientific Co.). The columns were perfused via the main port with modified Krebs-indomethacin solution. The BAECs were stimulated to release EDRF by UK14304 (from 10^-10 to 10^-5 M), BK (from 10^-11 to 10^-4 M), and A23187 (from 10^-10 to 10^-3 M) administered into the perfuse proximal to the main port by means of an infusion pump (model 901, Harvard Apparatus, South Natick, Mass.) at a rate of 0.2 ml/min. The release of EDRF was monitored as a decrease in bioassay ring tension and expressed as percent relaxation of active tension. Confirmatory evidence for EDRF release was obtained by the addition of methylene blue (10 μM) and hemoglobin (10 μM), substances that are known to inhibit the effects of EDRF, to the perfusate of the second port. Similarly, superoxide dismutase (150 units/ml) was added to the second infusion port to determine whether it potentiated the effects of EDRF.

Initial studies using UK14304, the α5-adrenergic agonist, were performed in the presence of (−)-propranolol (0.1 mM), prazosin (0.1 mM), and ascorbic acid (0.1 mM) with prostanandin F2o (4 μM) used to produce active tension. Preliminary results showed no effect of prazosin on UK14304-stimulated EDRF release; hence, later studies used L-phenylephrine (5 μM) to produce active tension (administered via the second infusion port) in the absence of prazosin. Specific activation of the α5-adrenergic receptor by UK14304 was demonstrated by infusing the α5-adrenergic receptor antagonists (rauwolscine [0.1 μM], oxytometazoline [0.1 μM], or prazosin [1 μM]) directly onto the bioassay tissue (via the second infusion port) so as to block UK14304 stimulation. The specific BK receptor antagonists, des-Arg^9,Leu^7-BK (1 μM) and [Thi^2,D-Phe^7]-BK (1 μM), were infused via the second port during BK stimulation to determine which receptor subtype was being activated by BK.

Radioligand Binding Studies

Similar binding conditions previously outlined for the α5-adrenergic receptor and BK receptor were used. Radioligand binding studies for the α5-adrenergic receptor were performed with [3H]rauwolscine or [3H]UK14304 at 12 concentrations ranging from 10^-12 to 10^-8 M. Nonspecific binding was determined by the addition of 0.1 mM (−)-epinephrine (K0.1, 0.1 μM) to each sample. For the BK1 receptor, binding was performed with [3H]BK at 12 concentrations ranging from 10^-12 to 10^-8 M. Nonspecific binding was determined in the presence of 10 μM [Thi^2,D-Phe^7]-BK.

Identification of G Protein Subtypes and Isoforms

Candidate G proteins present in BAECs were identified by specific polyclonal rabbit antisera. The P, antisera recognize the internal amino acid sequence DRIAQPN (161-167) of Gaq. The P, antisera recognize the common carboxy-terminal sequence KENLKDCGLF (345-354 and 346-355) of Gq and Giq. The EC/2 antisera recognize the carboxy-terminal sequence KENKKECGLY (346-355) of Gq, but also cross-react with Gai, and Giq. The SW/1 antisera recognize the carboxy-terminal sequence common to the β subunit.

Proteins from partially purified BAEC membranes (50 μg) and various known amounts of recombinant α5 and α5 were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10% running, 4% stacking gel) according to the method of Laemmli. The proteins were electrophoretically transferred onto Immobilon-P and incubated overnight at 4°C with the specific antisera. The Immobilon-P was washed twice with blocking solution (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% Nonidet P-40, 2.5% bovine serum albumin fraction V, 2.5% nonfat dry milk, 0.0002% NaN3, pH 8.0) and exposed to goat anti-rabbit horseradish peroxidase antibody before autoradiography at 23°C for 1 minute. To determine that the 40-kd protein recognized by P, antisera was the result of a specific interaction with Gai, we performed initial Western blots with an excess of the specific peptide, KENLKDCLGF, to the P, antisera to demonstrate that we could block this reaction.

ADP-Ribosylation With [32P]NAD in BAEC Membrane Preparations

The protocol was derived from the method of Ribeiro-Neto et al11 with some modifications. Partially purified membranes (100 μg) from rat brain and PTX-treated and -untreated BAECs were suspended in diethiothreitol (2 mM), Tris-HCl (25 mM), EDTA (1 mM), GTP (0.5 mM), ATP (1 mM), thymidine (10 mM), DNase I (0.02 μg/ml), bovine serum albumin (7.5 μg per assay), and Lubrol-PX (1%) in a total volume of 100 μl.
The reaction was begun with the addition of \(^{32}\)P\text{NAD}\ (60 \mu M) and preactivated PTX (10 \mu g/ml). Preliminary studies using unlabeled NAD (60 \mu M) followed by \(^{32}\)P\text{NAD}\ (60 \mu M) indicated that complete ADP-ribosylation is achieved after 2 hours at 32°C. The reaction was terminated with 1 ml ice-cold trichloroacetic acid (20%) and centrifuged at 12,000g for 10 minutes. The resulting pellets containing ADP-ribosylated G proteins were then resolubilized in denaturing buffer containing Tris-HCl (125 mM, pH 6.8), SDS (4%), glycerol (20%), and 2-mercaptoethanol (10%) and placed in boiling water for 5 minutes. The samples and known molecular weight markers (Bethesda Research Laboratory) were separated by SDS-PAGE (10% running, 4% stacking gel) before autoradiography at \(-70°C\) for 72 hours.

**Data Analysis**

All values including graphic depictions with error bars are expressed as mean±SEM compared with controls and among separate experiments. The radioligand binding studies were analyzed by the methodology of Scatchard\(^{12}\) and the \textsc{ligand} program of Munson and Rodbard.\(^{13}\) The EDRF bioassays were performed simultaneously for each condition and its control. Paired and unpaired Student’s t tests were used to determine the significance of shifts in \(K_d\), \(K_a\), and EC\_50 and changes in specific binding and isometric tension (EDRF release). A significant difference was taken for values of \(p<0.05\).

**Results**

**Cell Culture and Bioassay Tissue**

Relatively pure BAEC cultures were confirmed by immunofluorescent staining with factor VIII antibodies. Endothelin antibodies also stained these cells. There were no observable adverse effects of PTX (100 ng/ml, 16 hours) on cellular morphology, growth, or immunofluorescent staining. Deendothelialized rat thoracic aorta failed to react directly in response to A23187, UK14304, and BK, indicating partial removal of the endothelium. Light and scanning electron microscopy also revealed the absence of endothelial cells in adjacent rubbed aortic segments used in the bioassay experiments (photographs not included).

**Characterization of Receptors on Endothelial Cells**

Specific binding of \(^{3}H\text{UK14304}\) to BAEC membranes was completely blocked by rauwolscine (1 \mu M), partially blocked by oxymetazoline (1 \mu M), and not blocked by prazosin (10 \mu M) (Figure 1A). This is consistent with the \(\alpha\text{-adrenergic receptor subtype, } \alpha_2M\).\(^{14}\) Specific binding of \(^{3}H\text{BK}\) was blocked by \([\text{Thi}^{5',8},\text{D-Phe}^7]\)-BK (10 \mu M) but not by des-Arg\(^8\),Leu\(^7\)-bradykinin (0.1 \mu M), indicating the absence of endothelial cells in adjacent rubbed aortic segments used in the bioassay experiments (photographs not included).

Scatchard and \textsc{ligand} analysis of \(^{3}H\text{rauwolscine saturation showed one binding site with a } K_d\ of 1.7±0.2 nM and a } B_{\text{max}}\ of 200±19 fmol/mg. Nonspecific binding at 5 nM of \(^{3}H\text{rauwolscine with excess unlabeled (-)-epinephrine was 58±7 fmol/mg. However, } \text{UK14304 saturation binding studies revealed the existence of two classes of binding sites: an HAB site (94±16 fmol/mg) with a } K_d\ of 0.22±0.07 nM and a low affinity site (110±14 fmol/mg) with a } K_d\ of 1.40±0.05 nM (Figure 2A). With the addition of GppNH\_p, only one affinity site (200±13 fmol/mg) with a } K_d\ of 1.40±0.13 nM was detected. Nonspecific binding at 5 nM of \(^{3}H\text{UK14304 with excess cold rauwolscine was } 30±6 \text{ fmol/mg.}

Similarly, \(^{3}H\text{BK saturation binding studies also revealed two classes of binding sites: an HAB site (34±2.7 fmol/mg) with a } K_d\ of 16±1.3 \text{ pM and a low affinity site (82±9.3 fmol/mg) with a } K_d\ of 780±67 \text{ pM (Figure 2B). The addition of GppNH\_p resulted in the loss of the HAB site, with the remaining site having a } K_d\ of 870±73 \text{ pM and a } B_{\text{max}}\ of 120±9.5 \text{ fmol/mg. Nonspecific binding at 5 nM of } \text{UK14304 with excess } [\text{Thi}^{5',8},\text{D-Phe}^7]-\text{BK was 9.5} \text{ fmol/mg. Thus, HAB (G protein–coupled) sites were represented 46% of the } \alpha_2\text{-adrenergic receptor and 29% of the } B_{\text{K}}\text{ receptor population.}

**Release of EDRF**

Results of four separate experiments found that both UK14304 and BK elicited concentration-dependent re-
Experiments were performed four times in duplicates with <10% variability.

The lines drawn through the data points were derived from the LIGAND program of Munson and Rodbard. Note that nearly all of the high affinity sites are abolished with GppNHp. Experiments were performed four times in duplicates with <10% variability.

Maximal relaxation of bioassay tissue with EC_{50} values of 25±3.2 and 1.2±0.1 nM, respectively. Maximal relaxation of bioassay tension elicited by UK14304 was not different from BK (1.7±0.2 versus 1.6±0.2 g, p=NS), and the effects of maximal UK14304 and BK were not additive. These responses were potentiated by superoxide dismutase when the perfusate reaching the bioassay tissue was delayed by more than 60 seconds and inhibited by methylene blue and hemoglobin (data not shown). The response curve to UK14304 stimulation was shifted rightward to an EC_{50} of 620±43 nM (p<0.01) with the addition of either 0.1 μM rauwolscine or 1.0 μM idazoxan (Figure 3A). There was little or no shift of the EC_{50} with prazosin. The BK_{1} antagonist des-Arg^{9}[Leu^{8}]-BK (1 μM) did not significantly affect the responses of BAECs to BK (EC_{50} 1.2±0.3 nM; p=NS). However, in the presence of the BK_{2} antagonist [Thi^{8},D-Phe^{7}]-BK (1 μM), the BK response curve shifted significantly rightward with an EC_{50} of 40±3.7 nM, indicating that BK was acting via the BK_{2} receptor (Figure 3B). Calcium ionophore A23187 elicited relaxation with an EC_{50} of 20±3.2 nM and was not affected by rauwolscine or [Thi^{8},D-Phe^{7}]-BK.

Forskolin (1 μM) and 8-bromo-cAMP (0.1 mM) did not inhibit UK14304-stimulated release of EDRF, indicating that this α-adrenergic receptor effect was not mediated via decreases in intracellular cAMP levels (data not shown).

**Effects of PTX on EDRF Release**

Pretreatment of BAECs with PTX caused a decrease in maximal relaxations to both UK14304 and BK. There was no further inhibition of EDRF release with pretreatment of PTX as high as 300 ng/ml and as long as 36 hours. The release of EDRF in response to UK14304 was more sensitive than BK to treatment with PTX, with

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**FIGURE 2.** Scatchard diagram of [^{3}H]UK14304 (panel A) and [^{3}H]-bradykinin (panel B) saturation binding studies in the absence (control) and presence of GppNHp (0.1 mM). The lines drawn through the data points were derived from the LIGAND program of Munson and Rodbard. Note that nearly all of the high affinity sites are abolished with GppNHp.

**FIGURE 3.** Curves showing response of vascular smooth muscle (% relaxation) to bovine aortic endothelial cells stimulated with UK14304 (panel A) and bradykinin (panel B). Control, absence of any antagonists; BK_{1} ANTAG, des-Arg^{9}[Leu^{8}]-bradykinin (a bradykinin receptor type-1 antagonist); BK_{2} ANTAG, [Thi^{8},D-Phe^{7}]-bradykinin (a bradykinin receptor type-2 antagonist). Concentrations were as follows (μM): rauwolscine 0.1, idazoxan 1, BK_{1} ANTAG 1, and BK_{2} ANTAG 1.
There were only small shifts in the $EC_{50}$ with both agonists. Note that most of the endothelium-derived relaxing factor release via UK14304 stimulation is mediated by pertussis toxin-sensitive $G$ proteins ($G_{i}$ proteins), whereas more than half of the endothelium-derived relaxing factor release via bradykinin stimulation occurs via pertussis toxin-insensitive $G$ proteins or mechanism(s) independent of $G$ proteins.

**Effects of PTX on Receptor–$G_{i}$ Protein Coupling**

Treatment with PTX produced a loss of HAB sites to both $\alpha_{2A}$-adrenergic receptors and BK$_2$ receptors at several concentrations of each ligand ranging from 10 to 1,000 pM. This was best appreciated when the ligand binding studies were performed near the $K_{i}$ of the HAB site of either receptor. At 0.5 nM of $[^{3}H]$UK14304 (specific binding, 170±15 fmol/mg), PTX produced a loss of 56±3.2 fmol/mg of HAB sites as compared with GppNHP (78±5.3 fmol/mg) (Figure 5A). Similarly, at 0.05 nM of $[^{3}H]$BK (specific binding, 96±7.2 fmol/mg), loss of HAB sites after PTX and GppNHP treatment was 17±1.3 and 37±3.2 fmol/mg, respectively (Figure 5B). Thus, the loss of HAB sites indicates that PTX effectively uncouples 72% of the $G$ protein–coupled

**FIGURE 4.** Response curves showing effects of pertussis toxin (100 ng/ml) on endothelium-dependent relaxations to UK14304 (panel A) and bradykinin (panel B). Maximal inhibition of relaxation is greater with UK14304 than bradykinin. There were no small shifts in the $EC_{50}$ with both agonists.

**FIGURE 5.** Panel A: Bar graph showing percent of maximal relaxation in response to bradykinin (0.1 $\mu$M) and UK14304 (10 $\mu$M) with and without pertussis toxin pretreatment. There was no difference in maximal relaxation of controls between UK14304 and bradykinin. Panel B: Bar graph showing percent of high affinity agonist-binding sites (HABS) in bovine aortic endothelial cell membrane at 0.5 nM $[^{3}H]$UK14304 and 0.05 nM $[^{3}H]$-bradykinin with and without pertussis toxin pretreatment. There was complete loss of HABS for both agonists with the addition of GppNHP (0.1 mM). Experiments were performed four times in duplicate. *Significant (p<0.05) differences between control and pertussis toxin treatment. **Significant (p<0.05) differences in percent relaxation and percent specific binding between UK14304 and bradykinin.
\( \alpha_2 \)-adrenergic receptors but only 46% of the G protein–coupled BK receptor.

**Identification of G Protein Subtypes and Isoforms**

Western blot analysis of BAEC membranes with the P, and EC/2 antisera revealed single bands of 40 and 39 kd, respectively (Figure 6). Prior treatment with excess GGal carboxy-terminal peptide resulted in the loss of the 40-kd band that was previously recognized by the P, antisera (data not shown). The P, antisera, which are specific for GGal, failed to recognize a 40-kd protein in BAEC membranes but did recognize a protein of ~40 kd in rat brain. The EC/2 antisera also recognized in rat brain a protein of smaller molecular mass (39 kd) that was not present in endothelial cells. Therefore, our data indicate that BAEC membranes contain predominantly GGal and GGal but little, if any, GGal. Because EC/2 antibody may not recognize G, with the same affinity as GGal, we cannot completely exclude the presence of G, in BAECs by Western blotting using this antibody. When known amounts of recombinant \( \alpha_2 \), and \( \alpha_3 \) were used as standards on Western blots, the amount of GGal and GGal in BAEC membranes (100 ng) was 7.7±0.8 and 2.8±0.5 ng, respectively. This corresponded to 1.9 pmol GGal and 0.65 pmol GGal per milligram membrane protein. The concentration of the β subunit recognized by SW/1 antisera was unchanged in membranes from BAECs treated with PTX (data not included).

**\( ^{32} \text{P} \)/NAD Ribosylation of G Proteins**

The studies with \( ^{32} \text{P} \)/NAD–labeled membranes from rat brain revealed several bands: two bands running between 40 and 41 kd, consistent with G, protein(s), and another band running at 39 kd, which may represent G, proteins. In contrast, \( ^{32} \text{P} \)/NAD–labeled BAEC membranes revealed only a single band running between 40 and 41 kd, suggesting the absence of G, in BAEC membranes. After treatment with PTX, the BAEC membranes no longer contained the substrate for PTX–catalyzed \( ^{32} \text{P} \)/NAD ribosylation at 40–41 kd, indicating complete ADP-ribosylation of G, proteins.

**Discussion**

The release of EDRF in response to UK14304, an imidazoline derivative, appears to be mediated specifically via the \( \alpha_2 \)-adrenergic receptor since its response can be competitively blocked by the specific \( \alpha_2 \)-adrenergic receptor antagonist rauwolscine. The relative insensitivity to high concentrations of prazosin suggests that the probable subtype is \( \alpha_2A \). Most of the EDRF released in response to BK is mediated via BK receptor, since only the BK receptor antagonist [Thi144,α2Phe]–BK produced competitive inhibition. The relaxation of the vascular smooth muscle ring was due to a
labile nonprostanoid substance that was potentiated by superoxide dismutase and inhibited by methylene blue and hemoglobin. This is characteristic of endothelium-derived nitric oxide or a nitric oxide derivative that relaxes vascular smooth muscle by stimulating soluble guanylate cyclase.\(^\text{17,18}\)

Our results suggest that the release of EDRF via the \(\alpha_2\)-adrenergic receptor and the BK receptor is differentially coupled to \(G\) proteins in BAEC membranes. The \(\alpha_2\)-adrenergic receptor pathway appears to be more sensitive to PTX with respect to EDRF release compared with the BK receptor pathway since PTX-sensitive \(G\) proteins and HAB sites account for about 80\% of EDRF released via the \(\alpha_2\)-adrenergic receptor, whereas they account for less than half of the EDRF released via BK receptors. This is consistent with previous findings that BK-stimulated increase in inositol trisphosphate turnover and EDRF release in bovine pulmonary artery endothelial cells are relatively insensitive to PTX, although GDP\(\beta\)S could effectively block this activity.\(^\text{2}\)

Although two distinct binding sites have been reported for the BK receptor in bovine pulmonary artery endothelial cells, it is not known whether the higher affinity site represented \(G\) protein–coupled receptors.\(^\text{19}\) Similar pharmacological studies with the \(\alpha_2\)-adrenergic receptor have not been performed in BAECs, although HAB sites for both the \(\alpha\) and \(\beta\)-adrenergic receptor resulting from receptor–\(G\) protein coupling have been documented in other cell lines.\(^\text{20}\) Receptor binding agonists with high affinity reflect a productive interaction with \(G\) proteins.\(^\text{8}\) These sites are converted to ones of low affinity when uncoupling from their \(G\) protein is affected, as by the addition of guanine nucleotides or PTX treatment. The fact that a greater percentage of \(\alpha_2\)-adrenergic receptors are coupled to a PTX-sensitive \(G\) protein in BAECs may be a function of their preferential coupling to \(G\) proteins. This is consistent with our finding that more HAB sites are produced and a greater amount of EDRF is released via PTX-sensitive \(\alpha_2\)-adrenergic receptor than BK receptor pathways.

Receptor–\(G\) protein coupling may be influenced by several factors that include receptor phosphorylation, colocalization of receptors and \(G\) proteins with putative membrane domains, and the availability of \(G\) proteins for receptor coupling. Furthermore, PTX-catalyzed ADP-ribosylation of the \(\alpha\) subunit of \(G\) proteins is a complex process whose efficiency is likely determined by stoichiometry, accessibility,\(^\text{2\,1}\) posttranslational modification,\(^\text{2\,1}\) and affinity for \(\beta\)Y, which is required for efficient ADP-ribosylation.\(^\text{2\,1}\) It is curious that only 29\% of the BK receptor population is \(G\) protein–coupled in BAEC membranes as compared with ~50\% of the \(\alpha_2\)-adrenergic receptor population. This difference may reflect the differing abilities of these receptors to couple to the repertoire of \(G\) proteins available in BAECs. For example, among three \(G\) protein isoforms previously identified, we have shown that BAEC membranes contain only two of these isoforms, \(G_{\alpha_2}\) and \(G_{\alpha_3}\).\(^\text{2\,3}\) These isoforms may possess both differential affinities for their receptor(s) and sensitivities to PTX. Hence, the greater percentage of HAB sites observed with the \(\alpha_2\)-adrenergic receptor may be due to its more efficient coupling to \(G_{\alpha_2}\) which appears to be the predominant PTX-sensitive substrate in BAEC membranes.

The \(\alpha_2\)-adrenergic receptor in BAECs appears to couple rather selectively to \(G_\alpha\) proteins in signaling the release of EDRF, whereas the BK receptor can effectively couple to both PTX-sensitive and \(-\)insensitive \(G\) proteins. Such specificity and diversity in signal transduction pathways have been documented in other cell types. Multiple \(G\) proteins that activate phospholipase \(C\) are selectively coupled to different receptors in Chinese hamster ovary cells and are also distinguished by their PTX sensitivity.\(^\text{2\,4}\) The dopamine \((D_\text{2})\) receptor selectively couples to \(G_{\alpha_2}\) in reconstituted phospholipid vesicles.\(^\text{2\,5}\) Treatment of platelet and neuroblastoma glioma–hybridoma cell membranes with specific carboxyterminal–directed \(G_{\alpha_2}\) antisera blocks \(\alpha_2\)-adrenergic and \(\delta\)-opioid receptor–mediated inhibition of adenyl cyclase.\(^\text{2\,6,2\,7}\) Similarly, the release of EDRF can be mediated via diverse transduction pathways with specific receptors either using or sharing distinct \(G\) proteins.

In summary, the release of an important physiological vasodilator, EDRF, from BAECs is mediated by selective receptor–\(G\) protein coupling. Our data indicate that EDRF release via the \(\alpha_2\)-adrenergic receptor is more closely coupled to \(G\) proteins than release via BK receptors in BAECs, whereas coupling of the BK receptor is accomplished predominantly through PTX-insensitive \(G\) proteins. Specificity at the level of receptor–\(G\) protein interaction may provide the endothelial cell with mechanism(s) to modulate EDRF release at or beyond the level of the hormone–receptor interface. These processes that direct the traffic of cell surface signals may involve not only the type of receptor but also the particular \(G\) protein to which it is coupled. It remains to be determined whether modulation of both the amount and the expression of specific \(G\) protein isoforms can influence the release of EDRF via these two receptor pathways in endothelial cells.

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