JCI The Journal of Clinical Investigation

The G proteins of the G alpha i and G alpha q family couple the bradykinin receptor to the release of endothelium-derived relaxing factor.

J K Liao, C J Homcy

J Clin Invest. 1993;92(5):2168-2172. https://doi.org/10.1172/JCI116818.

Research Article

Bradykinin stimulates diverse functions in endothelial cells including the release of endothelium-derived relaxing factor (EDRF). Little is known, however, regarding the identity of the G protein(s) involved. Here we demonstrate that G proteins of the G alpha i and G alpha q family are coupled to the bradykinin receptor (BKR) in bovine aortic endothelial cells by using specific antisera directed against the COOH-terminal region of G alpha i2 (P4), G alpha i3 (EC), and G alpha q (QL). These antisera are specific since their effects are blocked by the decapeptides from which they were derived. The degree of receptor-G protein coupling was assessed by the formation of high affinity agonist binding sites (HABS) and GTP hydrolysis. In a concentration-dependent manner, the QL antisera reduced HABS and GTPase activity by 65 and 60%, respectively, and effectively abolished them in membranes from pertussis toxin-treated cells. The combination of P4 and EC antisera produced a loss of HABS (41%) and GTPase activity (40%) comparable to the effects of pertussis toxin. These findings indicate that G alpha i and G alpha q proteins mediate the cellular responses to bradykinin in bovine aortic endothelial cells and support the observation that bradykinin-stimulated EDRF release is relatively insensitive to pertussis toxin.

Find the latest version:

https://jci.me/116818/pdf

The G Proteins of the $G\alpha_i$ and $G\alpha_q$ Family Couple the Bradykinin Receptor to the Release of Endothelium-derived Relaxing Factor

James K. Liao and Charles J. Homcy

Cardiovascular Division, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; the Department of Medicine, College of Physicians & Surgeons of Columbia University, New York, New York 10032; and the Medical Research Division, American Cyanamid Company-Lederle Laboratories, Pearl River, New York 10965

Abstract

Bradykinin stimulates diverse functions in endothelial cells including the release of endothelium-derived relaxing factor (EDRF). Little is known, however, regarding the identity of the G protein(s) involved. Here we demonstrate that G proteins of the $G\alpha_i$ and $G\alpha_q$ family are coupled to the bradykinin receptor (BKR) in bovine aortic endothelial cells by using specific antisera directed against the COOH-terminal region of $G\alpha_{i2}$ (P4), $G\alpha_{i3}$ (EC), and $G\alpha_q$ (QL). These antisera are specific since their effects are blocked by the decapeptides from which they were derived. The degree of receptor-G protein coupling was assessed by the formation of high affinity agonist binding sites (HABS) and GTP hydrolysis. In a concentration-dependent manner, the QL antisera reduced HABS and GTPase activity by 65 and 60%, respectively, and effectively abolished them in membranes from pertussis toxin-treated cells. The combination of P4 and EC antisera produced a loss of HABS (41%) and GTPase activity (40%) comparable to the effects of pertussis toxin. These findings indicate that $G\alpha_i$ and $G\alpha_q$ proteins mediate the cellular responses to bradykinin in bovine aortic endothelial cells and support the observation that bradykinin-stimulated EDRF release is relatively insensitive to pertussis toxin. (*J. Clin. Invest.* 1993. 92:2168–2172.) Key words: pertussis toxin • carboxy-terminus antisera • high affinity agonist binding sites • GTPase activity • Western Blotting

Introduction

Bradykinin plays an important role in vascular proliferation, permeability, and relaxation (1–3). Its various actions are mediated by membrane receptors coupled to heterotrimeric G proteins in endothelial and vascular smooth muscle cells (4, 5). We and others have previously shown that stimulation of bradykinin receptors in bovine aortic endothelial cells causes the release of endothelium-derived relaxing factor (EDRF)[1] (6, 7). The signaling pathway(s) is relatively insensitive to pertussis toxin, and involves hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and mobilization of intracellular calcium (8, 9). The G_i proteins can couple the bradykinin receptor to EDRF release, but the identity of the actual G_i protein isoforms (i.e., $G\alpha_{i1,2,3}$) mediating this response have not been clearly established. Furthermore, since most of the EDRF release is resistant to the effects of pertussis toxin, the primary mediator of phospholipase C activation and EDRF release via the bradykinin receptor is not the G_i family of proteins, but a more vaguely defined class of G proteins previously referred to as G_p (10).

Recently, a family of 42-kD G proteins, called G_q, have been shown to couple the vasopressin, angiotensin, and thromboxane A_2 receptor to the activation of phospholipase C (11–13). This is intriguing since these G_q proteins lack the COOH-terminal cysteine residue required for covalent modification by pertussis toxin and their tissue distribution and ability to activate phospholipase C appear to be similar to that described for G_p (14, 15). In fact, the stimulation of PIP_2 hydrolysis by bradykinin has been shown to be pertussis toxin-insensitive and inhibited by COOH-terminal α_q antisera in NG108-15 cells (12). However, the existence and function of G_q proteins in endothelial cells have not been demonstrated. We hypothesized that in addition to G_i proteins, G proteins of the G_q family are involved in coupling the bradykinin receptor to EDRF release. This potentially may be important since unlike G_i proteins, bradykinin-stimulated EDRF release mediated by pertussis toxin-insensitive G proteins appears to be relatively unaffected by endothelial trauma, hypercholesterolemia, and early atherosclerosis (16–18).

The COOH-terminal region of G proteins is the putative site of receptor interaction (19). Blocking this recognition site by pertussis toxin-catalyzed ADP-ribosylation or by COOH-terminal directed G protein antisera leads to receptor-G protein uncoupling as assessed by a loss of high affinity agonist receptor sites and a decrease in hormone-stimulated GTP hydrolysis (6, 20, 21). The COOH-terminal directed G protein antisera have become a very useful tool for determining specificity in receptor–G protein interactions. In this study, we used a similar approach to define and characterize the pertussis toxin-sensitive and -insensitive G proteins, which couple the bradykinin receptor to EDRF release.

Methods

Materials. All tissue culture reagents, unless otherwise stated, were obtained from JRH Bioscience. Bradykinin, [Thi^{5,8},D-Phe^7]-bradykinin (BKR antagonist), pertussis toxin, guanosine 5′-(β,γ-imidotriphosphate) (GppNHp), phenylmethylsulfonyl fluoride (PMSF), creatinine phosphate, phosphocreatine kinase, ATP, GTP, bacitracin, leupeptin, aprotinin, 1,10-phenanthroline, triethanolamine HCl, polyethyleneimine, DTT, and BSA were purchased from Sigma Chemical

Address correspondence to Dr. James K. Liao, Cardiovascular Division, Department of Medicine, Brigham & Women's Hospital, 221 Longwood Avenue (LMRC-307), Boston, MA 02115.

Received for publication 12 April 1993 and in revised form 4 June 1993.

1. *Abbreviations used in this paper:* BKR, bradykinin receptor; EDRA, endothelium-derived relaxing factor; HABS, high affinity agonist binding sites; PIP_2, phosphatidylinositol 4,5-bisphosphate.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/93/12/2168/05 $2.00
Volume 92, November 1993, 2168–2172

Co., (St. Louis, MO). [^{3}H] Bradykinin (121.6 Ci/mmol), and [γ-^{32}P]-GTP (10 Ci/mmol) were supplied by New England Nuclear (Boston, MA). The polyclonal rabbit COOH-terminal antisera P4 ($G\alpha_{i2}$) and CH5 ($G\alpha_s$) were raised in our laboratory. The EC ($G\alpha_{i3}$), QL ($G\alpha_q$), and SW (common β subunits) antisera were purchased from Dupont Co. (Wilmington, DE). Purified decapeptides corresponding to the COOH-terminal region of $G\alpha_{i2}$ (P4 peptide, KENLKDCGLF), $G\alpha_{i3}$ (EC peptide, KENLKECGLY), $G\alpha_q$ (QL peptide, QLNLKEYNLV) were synthesized by Research Genetics, Inc. (Huntsville, AL). Protein molecular weight markers were purchased from Bethesda Research Laboratories, Inc. (Bethesda, MD). The Western Blotting kit (ECL) using horseradish peroxidase and luminol was obtained from Amersham Corp. (Arlington Heights, IL). Westran was purchased from Schleicher and Schuell (W. Germany).

Cell culture. Bovine aortic endothelial cells were harvested and cultured at 37°C in a growth medium containing DME, 5 mM L-glutamine (Gibco Laboratories, Grand Island, NY) 10% FCS (Hyclone Laboratories, Logan, UT) and penicillin (100 U/ml)/streptomycin (100 mg/ml)/Fungizone (250 ng/ml). They were characterized by Nomarski optical microscopy (Zeiss ICM 405, 40× objective) and immunofluorescence staining with anti-Factor VIII antibodies. All cell passages were performed with a disposable cell scraper (Costar, Cambridge, MA) and not with trypsin, and only endothelial cells of less than 3 passages were used. Partially purified membranes from control and pertussis toxin-treated cells (100 ng/ml, 16 h) were prepared as previously described (6).

Western blotting of G_i and G_q proteins. Membrane proteins (50 μg) were separated by SDS/polyacrylamide gel electrophoresis (10% running, 4% stacking gel) according to the method of Laemmli (22). The proteins were electrophorectically transferred onto Western and incubated separately overnight at 4°C with P4 (1:200), EC (1:1,000), and QL antisera (1:1,000) in the presence and absence of excess (10 μg/ml) COOH-terminal decapeptides. The Westran was washed twice with blocking solution (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% NP-40, 2.5% BSA-fraction V, 5% nonfat dry milk, pH 8.0) and exposed to donkey anti-rabbit horseradish peroxidase antibody (1:2,000), before autoradiography at 23°C for 1 min. Specificity of the protein band was determined by its size and the ability of specific COOH-terminal peptides to block its corresponding antisera recognition.

Radioligand binding studies. Membranes (100 μg) were incubated with serial dilutions of QL antisera (1:10 to 1:2,000), P4 antisera (1:50 to 1:1,000), EC antisera (1:50 to 1:1,000), and CH5 antisera (1:50) in the presence and absence of competing QL, P4, and EC decapeptides (0.1 to 10 μg/ml) for 60 min at room temperature (22°C). The mixture was then added to the binding buffer containing Tris-HCl (100 mM), $MgCl_2$ (5 mM), EDTA (0.6 mM), bacitracin (140 μg/ml), captopril (1 μM; Squibb & Sons, Inc., Princeton, NJ), DTT (1 mM), leupeptin (100 μg/ml), aprotinin (50 μg/ml), 1,10-phenanthroline (1 mM), and BSA (0.1%), pH 7.4 in a total volume of 0.1 ml. The assay was initiated by the addition of 60 pM of [^{3}H]bradykinin to the reaction mixture. We have previously determined that the K_i of the high-affinity bradykinin receptor site in endothelial membrane is 36 pM with a low-affinity site K_i of 1.4 nM (6).

The assay was shaken vigorously for 90 min at 4°C and then terminated by vacuum-filtration through GF/C filters (Whatman Inc., Clifton, NJ). Each filter containing receptor-ligand complex was counted for 2 min in a liquid scintillation counter (model LS 1800; Beckman Instruments, Inc., Fullerton, CA). All reaction tubes and filters were pretreated overnight with 0.1% BSA and polyethyleneimine. Nonspecific binding (100–150 CPM) was determined in the presence of 1 μM [Thi^{5,8},D-Phe^7]bradykinin. Low affinity binding was determined in the presence of 10 μM GppNHp and represented 20–25% of total specific binding. BKR HABS was calculated as the difference between total and low affinity binding. Assays were performed four times in triplicate.

GTPase assay. The protocol used to measure agonist-stimulated GTPase activity was derived from the method used by Shenker et al. with some modifications (13). Assay conditions consisted of [γ-^{32}P]-GTP (0.5 μM), $MgCl_2$ (5 mM), EGTA (0.1 mM), NaCl (50 mM), creatine phosphate (4 mM), phosphocreatine kinase (5 units), ATP (0.1 mM), DTT (1 mM), bacitracin (140 μg/ml), captopril (1 μM; E. R. Squibb & Sons, Inc.), PMSF (0.1 mM), leupeptin (100 μg/ml), aprotinin (50 μg/ml), 1,10-phenanthroline (1 mM), BSA (0.2%), and triethanolamine HCl (50 mM), pH 7.4, in a total volume of 0.1 ml with various concentrations of bradykinin (1 to 100 nM). Membranes (30 μg) were initially incubated at 22°C for 60 min in the presence and absence of indicated antisera and oligopeptides. The assay was initiated by adding the membranes to the reaction mixture and incubating for an additional 15 min at 22°C. The reaction was terminated with 500 μl of ice-cold 5% activated charcoal in phosphoric acid (50 mM) and the mixture was centrifuged for 10 min at 12,000 *g* at 4°C. The liberated $^{32}P_i$ was determined by counting the supernatant for 1 min in a liquid scintillation counter. Nonspecific activity was determined in the presence of 0.1 mM of unlabeled GTP. Spontaneous release of $^{32}P_i$ and low affinity GTPase activity (non-agonist-stimulated) accounted for only 0.4 and 29% of total specific activity, respectively. High affinity (bradykinin-stimulated) GTPase activity was calculated as the difference between total and low affinity GTPase activity. Assays were performed four times in triplicate.

Data analysis. All values are expressed as mean±SEM compared to controls and among separate experiments. Paired and unpaired Student's *t* tests were employed to determine the significance of changes in HABS and GTPase activity. A significant difference was taken for *P* values less than 0.05.

Results

Demonstration of $G\alpha_i$ and $G\alpha_q$ subunits in endothelial cells. Of the possible pertussis toxin-sensitive G proteins available for receptor coupling, we have previously shown by Western blotting that bovine aortic endothelial cell membranes contain predominantly $G_{\alpha i2}$ and $G_{\alpha i3}$, with little if any $G_{\alpha i1}$ or $G_{\alpha 0}$ (6). Recognition of $G_{\alpha i2}$ and $G_{\alpha i3}$ by P4 and EC antisera was completely blocked only by their corresponding decapeptides, respectively (Fig. 1). Similarly, the QL antisera recognized a 42-kD protein band in endothelial membranes. This recognition was specific, since in the presence of increasing concentrations of QL peptide (QLNLKEYNLV), but not P4 (KENLKDCGLF) and EC (KENLKECGLY) peptide, the QL antisera no longer recognized this 42-kD band. In addition, membranes from pertussis toxin–treated cells did not show significantly different amount of α_i, α_q, α_s and common β subunit compared to control membranes (data not shown).

The effects of COOH-terminal G protein antisera on BKR HABS and GTPase activity. Stimulation by bradykinin resulted in the formation of 34.5±2.3 fmol/mg of BKR HABS and 15.2±1.2 pmol/min per mg of GTP hydrolysis. The low affinity BKR sites and GTPase activity as defined in the pres-

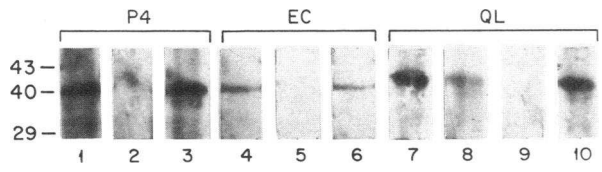

Figure 1. Western blots of endothelial cell membranes (50 μg) treated with the P4 ($G\alpha_{i2}$), EC ($G\alpha_{i3}$), and QL ($G\alpha_q$) antisera in the presence or absence of the indicated oligopeptides. Lanes *1,4,* and *7* are controls. Lane *2* shows P4 peptide (10 μg/ml). Lane *3* shows a combination of EC and QL peptides (10 μg/ml each). Lane *5* shows EC peptide (10 μg/ml). Lane *6* shows a combination of P4 and QL peptide (10 μg/ml each). Lane *8* shows QL peptide (1 μg/ml). Lane *9* shows QL peptide (10 μg/ml). Lane *10* shows a combination of P4 and EC peptide (10 μg/ml each).

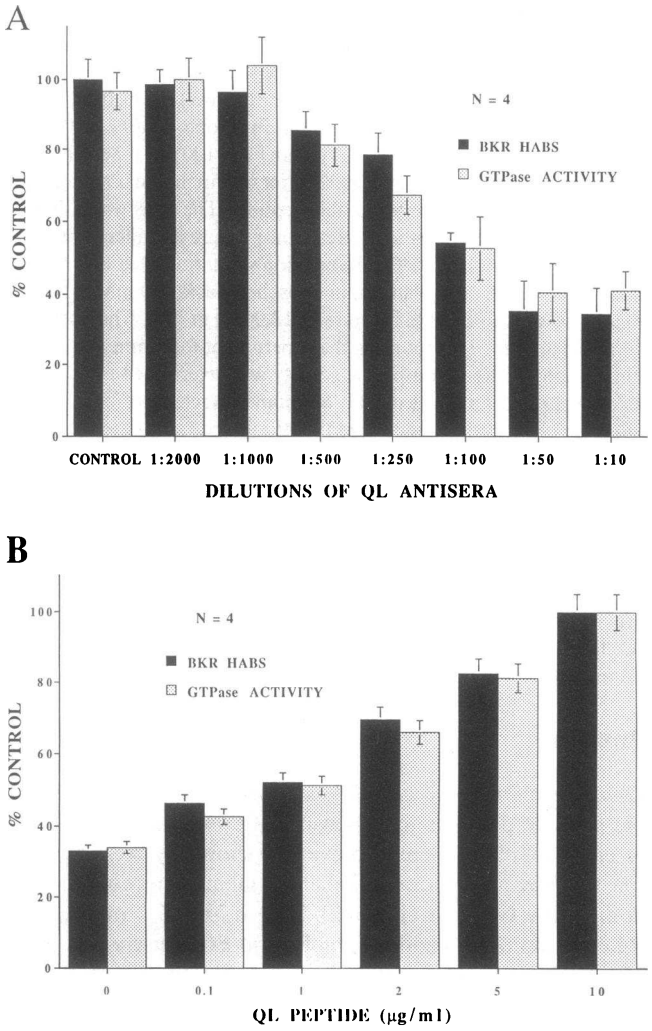

Figure 2. (*A*) The effects of increasing concentrations (decreasing dilutions) of QL antisera on bradykinin-stimulated BKR HABS formation and GTPase activity as percent of control (no antisera). Baseline bradykinin-stimulated BKR HABS and GTPase activity were 34.5 fmol/mg and 15.2 pmol/min/mg, respectively. Agonist-stimulated HABS formation and GTPase activity were calculated as the difference between total and low affinity values, respectively. (*B*) The effects of increasing concentrations of QL peptide ($G\alpha_q$, QLNLKEYNLV) on high affinity agonist binding sites (BKR HABS) and GTP hydrolysis (GTPase activity) in membranes treated with the QL antisera (1:50 dilution). The control membrane denotes absence of oligopeptides.

ence of GppNHp were 8.3±3.4 fmol/mg and 5.7±0.5 pmol/min per mg, respectively. The QL antisera caused a concentration-dependent parallel decrease in BKR HABS and high affinity GTPase activity (Fig. 2 *A*). At a 1:50 dilution, it reduced the number of BKR HABS by 65% (11.4±1.8 fmol/mg) and the level of GTPase activity by 60% (5.5±0.9 pmol/min per mg). The lack of complete abolition of BKR HABS and GTPase activity by the QL antisera suggests that G proteins other than $G\alpha_q$ can couple the bradykinin receptor. The inhibition of BKR HABS and GTPase activity by the QL antisera was specifically reversed by preincubation with increasing amounts of QL (QLNLKEYNLV) peptide (Fig. 2 *B*). The P4 (KENLKDCGLF) or EC (KENLKECGLY) peptide did not block the effects of the QL antisera on HABS formation (10.2±1.3 and 11.8±2.0 fmol/mg) and GTPase activity (5.5±0.7 and 4.9±0.8 pmol/min per mg), respectively. A similar concentration-dependent reduction of BKR HABS and GTPase activity was observed with the P4 and EC antisera. Both of these antisera produced a comparable decrease in BKR HABS (19.3%±5.4% vs. 26.5%±2.3%, P = NS) and GTPase activity (25.3%±7.3% vs. 24.7%±6.0%, P = NS), and in combination, lowered the BKR HABS and GTPase activity by 40.6±3.3 and 40.0±4.0% (P = NS) (Fig. 3). The effects of the antisera were specific since, in the presence of excess peptides from which they were derived, their inhibitory effects were essentially blocked. The combination of the QL, P4, and EC antisera produced a complete loss of BKR HABS (1.2±8.9 fmol/mg) and nearly abolished all of the bradykinin-stimulated GTPase activity (3.1±0.6 pmol/min per mg).

Membranes from pertussis toxin-treated endothelial cells show a 43% loss of BKR HABS (19.7±2.3 fmol/mg) and a 47% decrease in high affinity GTPase activity (7.1±0.9 pmol/min per mg). These results are comparable to the combined effects of the P4 and EC antisera in control membranes, although pertussis toxin appears to be slightly more effective in decreasing high affinity GTPase activity (Fig. 4). Neither the P4, EC, nor a combination of these two antisera caused a further loss of BKR HABS or decrease in high affinity GTPase activity. The QL antisera, however, was quite effective in abolishing all of the BKR HABS (0±5.3 fmol/mg) and high-affinity GTPase activity (1.2±0.6 pmol/min per mg) in membranes from pertussis toxin-treated cells.

Discussion

In this study, we have identified a pertussis toxin-insensitive G protein family, G_q, in bovine aortic endothelial cell membranes, and demonstrated that it can effectively couple to the bradykinin receptor. The bradykinin receptors in bovine aortic endothelial cells have been previously shown to be of only one pharmacologic subtype, B_2 (6). Uncoupling of G_q by COOH-terminal QL antisera produces a functional loss of 60–65% of BKR HABS and bradykinin-stimulated GTP hydrolysis. This effect is specific since the inhibitory effect of the QL antisera is concentration-dependent and reversed only by the presence of

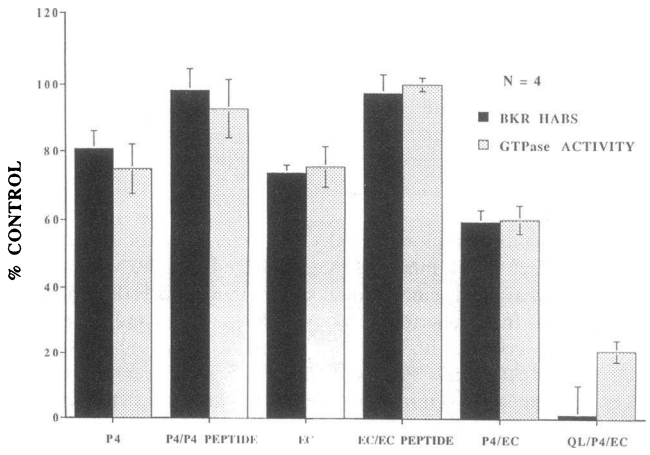

Figure 3. Membranes treated with the indicated antisera (1:50 dilution) and oligopeptides (10 μg/ml) were stimulated with bradykinin. Receptor-G protein coupling was assessed by high affinity agonist binding sites (BKR HABS) and GTP hydrolysis (GTPase activity), and expressed as percent of control (absence of both antisera or oligopeptide).

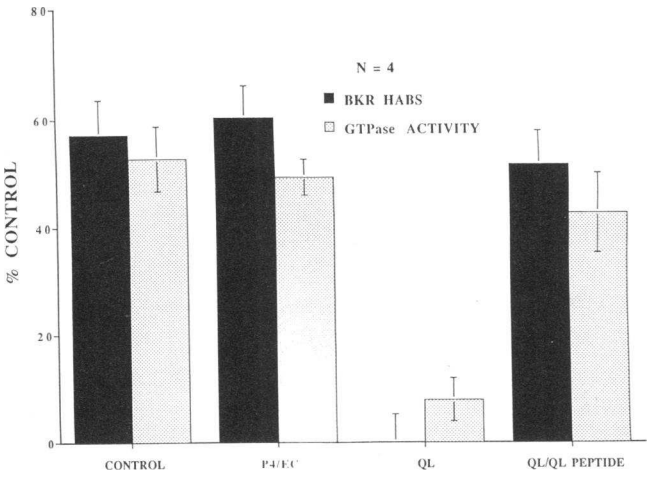

Figure 4. Membranes from pertussis toxin-treated cells (100 ng/ml) incubated with the indicated antisera and oligopeptide before bradykinin stimulation.

excess decapeptide to which the antisera is directed. These results are in stoichiometric agreement with our previous finding that nearly 60% of the bradykinin-stimulated EDRF release occurs via a pertussis toxin-insensitive pathway(s), and established G_q as the most likely, if not the only candidate, for mediating this response.

The G_q family consists of $G\alpha_{q/11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$ (23–25). No distinction is made between $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$ in this study since the COOH-terminal decapeptide sequence of $G\alpha_q$ and $G\alpha_{11}$ are identical and differs from $G\alpha_{14}$ by only two amino acids. The COOH-terminal decapeptide sequence of $G\alpha_{15/16}$, however, is considerably different, and in previous studies is not recognized by the COOH-terminal antisera to $G\alpha_{q/11}$ (25). Thus, we cannot exclude the possibility that the inhibitory effects of the QL antisera were not directed against several members of the G_q family. Other pertussis toxin-insensitive G proteins, which are known to activate phospholipase C, such as $G\alpha_z$ or $G\alpha_h$, could be involved in bradykinin-stimulated EDRF release (26, 27). However, their roles appear to be minimal or none, given that the QL antisera alone was able to abolish all BKR HABS and GTPase activity in membranes from pertussis toxin-treated endothelial cells.

The pertussis toxin-sensitive component of the bradykinin-stimulated EDRF release pathway is mediated by $G\alpha_{i2}$ and $G\alpha_{i3}$. These isoforms are the only detectable pertussis toxin-sensitive G protein substrate found in endothelial cells, and their effects on HABS formation and GTP hydrolysis can be specifically blocked by the P4 and EC antisera (6, 28). The degree of receptor-G protein uncoupling as assessed by high affinity agonist binding sites caused by the combination of these two antisera was similar to that caused by pertussis toxin. Pertussis toxin, however, appears to be slightly more effective in decreasing GTPase activity. This is probably due to a small difference in the degree of receptor-G_i protein uncoupling observed with covalent modification by pertussis toxin in the intact cell compared to competitive COOH-terminal recognition by the P4 and EC antisera in partially-purified membranes. Functionally however, $G\alpha_{i2}$ and $G\alpha_{i3}$ account for most of the pertussis toxin-sensitive coupling and each appears to be equivalent in its ability to couple the bradykinin receptor despite an almost threefold excess of $G\alpha_{i2}$ compared to $G\alpha_{i3}$ in bovine aortic endothelial cells (28). This suggests that $G\alpha_{i3}$ may have a higher affinity for, or tend to co-localize with, the bradykinin receptor in the cellular membrane.

The COOH-terminus of the G protein α subunit is the putative site of interaction with receptors (19). Blocking this site with a specific COOH-terminal antibody has been shown to cause receptor-G protein uncoupling and inhibition of receptor-mediated responses (29). The COOH-terminal $G\alpha_{i2}$-specific antisera has been used to block α_2-adrenergic and δ-opioid receptor-mediated inhibition of adenylyl cyclase in platelets and NG108-15 cells (30, 31). Antibodies that recognize the COOH-terminus of α_q subunits cause attenuation of phosphatidylinositide 4,5-bisphosphate hydrolysis when stimulated with bradykinin, angiotensin, histamine, and thyroid-releasing hormone in NG108-15 cells, rat liver, 1321N1 cells, and GH_3 rat pituitary cells (12, 25). Although this approach to affecting receptor-G protein uncoupling is limited to in vitro membrane preparations, it has greater specificity and applicability than pertussis toxin because both pertussis-toxin-sensitive and -insensitive α subunits can be targeted. A further consequence of receptor-G protein uncoupling is the diminution of subsequent guanine nucleotide exchange and hydrolysis on the α subunit (21). Such reduction in agonist-stimulated GTPase activity was observed in platelet membranes treated with α_q-specific COOH-terminal antisera before the stimulation of thromboxane A_2 receptors (13). Similarly, we were able to demonstrate different degrees of receptor-G protein uncoupling via the loss of high affinity agonist binding sites and decrease in agonist-stimulated GTPase activity using the COOH-terminal antisera to $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{q/11}$. This approach for determining receptor-G protein specificity, therefore, is applicable in many systems.

Although functional receptor coupling was blocked by the effects of pertussis toxin and α subunit-specific COOH-terminal antisera, we cannot exlude the possible indirect effects that pertussis toxin and these antisera could have on the function of associated $\beta\gamma$ subunits. This may be important since specific $\beta\gamma$ subunits have been shown to activate potassium channels and stimulate phospholipase C activity (32–34). Thus, one possible explanation for our findings is that by uncoupling the bradykinin receptor from its G proteins, more $\beta\gamma$ subunits are shifted into the inactive heterotrimeric configuration with the α subunit and less are available for stimulating effector activity. This heterotrimeric association is also required for efficient ADP-ribosylation of the α subunit by pertussis toxin (35). Indeed, we have found that $G\alpha_{i2}$ is more sensitive to ADP-ribosylation by pertussis toxin than $G\alpha_{i3}$ in endothelial cell membranes (28). This increased sensitivity to pertussis toxin may be due to specific $\beta\gamma$ subunit isoforms present in bovine aortic endothelial cells, which have a higher affinity for α_{i2} compared to α_{i3}. Thus, factors such as $\beta\gamma$ subunit composition, availability, and affinity for α subunits and effectors may all play a role in determining how signals are transduced across cellular membranes.

Of potential clinical importance is the observation that the release of EDRF via the pertussis toxin-sensitive pathway is altered by lipoproteins, early atherosclerosis, and trauma, while its release via the pertussis toxin-insensitive pathway is relatively unaffected (16–18). In porcine coronary artery and rabbit aorta, endothelial dysfunction (i.e., decrease in EDRF release) caused by oxidized LDL, lysophosphotidylcholine, or balloon injury, was initially observed with the serotonin, thrombin, and α_2-adrenergic receptor (36–38). These receptors are predominantly coupled to $G\alpha_i$ proteins. The minor

portion of these receptor signaling pathways that is pertussis toxin-insensitive shows no impairment in mediating EDRF release. Similarly, the stimulation of phospholipase C and EDRF release via the bradykinin receptor is relatively insensitive to treatment with both pertussis toxin and lipoproteins (6, 12, 16). In this study, we have shown that G proteins of the $G\alpha_q$ family are the primary mediator of bradykinin-stimulated EDRF release via the pertussis toxin-insensitive pathway(s). It is not known why the signaling pathway mediated by $G\alpha_q$, and not $G\alpha_i$, is spared in hypercholesterolemic states, and whether over-expression of $G\alpha_q$ in endothelial cells can prevent the progression of endothelial dysfunction.

In summary, we have identified and characterized the G protein components of both the pertussis toxin-sensitive and -insensitive bradykinin signaling pathways for EDRF release. The bradykinin receptor is predominantly coupled to pertussis toxin-insensitive $G\alpha_q$ although pertussis toxin-sensitive $G\alpha_{i2}$ and $G\alpha_{i3}$ can also contribute to this signaling pathway.

Acknowledgments

This work was supported by the National Institutes of Health grant HL02508, and by the Medical Research Division, American Cyanamid Company-Lederle Laboratories.

References

1. Goldstein, R. H., and M. Wall. 1984. Activation of protein formation and cell division by bradykinin and des-Arg^9-bradykinin. *J. Biol. Chem.* 259:9263–9268.

2. Murray, M. A., D. D. Heistad, and W. G. Mayhan. 1991. Role of protein kinase C in bradykinin-induced increase in microvascular permeability. *Circ. Res.* 68:1340–1348.

3. Ignarro, L. J., R. E. Byrns, G. M. Buga, and K. S. Wood. 1987. Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am. J. Physiol.* 253:H1074–1082.

4. Voyno-Yasenetskaya T. A., V. A. Trachuk, E. G. Cheknyova, M. P. Panchenko, G. Y. Grigorian, R. J. Vavrek, J. M. Stewart, and U. S. Ryan. 1989. Guanine nucleotide-dependent, pertussis toxin-insensitive regulation of phosphoinositide turnover by bradykinin in bovine pulmonary artery endothelial cells. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:44–51.

5. Tropea, M. M., D. Gummelt, M. S. Herzig, and L. M. Leeb-Lundberg. 1993. The B_1 and B_2 kinin receptors on cultured rabbit superior mesenteric artery smooth muscle cells: receptor-specific stimulation of inositol phosphate formation and arachnidonic acid release by des-Arg^9-bradykinin and bradykinin. *J. Pharmacol. Exp. Ther.* 264:930–937.

6. Liao, J. K., and C. J. Homcy. 1992. Specific receptor-guanine nucleotide binding protein interaction mediates the release of endothelium-derived relaxing factor. *Circ. Res.* 70:1018–1026.

7. Flavahan, N. A., H. Shimokawa, and P. M. Vanhoutte. 1989. Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. *J. Physiol.* 408:549–560.

8. Tkachuk, V. A., and T. A. Voyno-Yasnetskaya. 1991. Two types of G proteins involved in regulation of phosphoinositode turnover in pulmonary endothelial cells. *Am. J. Physiol.* 261:118–122.

9. Loeb, A. L., N. J. Izzo, R. M. Johnson, J. C. Garrison, and M. J. Peach. 1988. Endothelium-derived relaxing factor release associated with increased endothelial cell inositol trisphosphate and intracellular calcium. *Am. J. Cardiol.* 62:36–40.

10. Pang, I.-H., and P. C. Sternweis. 1990. Purification of unique alpha subunits of GTP-binding regulatory proteins (G proteins) by affinity chromatography with immobilized $\beta\gamma$ subunits. *J. Biol. Chem.* 265:18707–18712.

11. Wange, R. L., A. V. Smrcka, P. C. Sternweis, and J. H. Exton. 1991. Photoaffinity labeling of two rat liver plasma membrane proteins with [^{32}P]γ-azidoanilido GTP in response to vasopressin. *J. Biol. Chem.* 266:11409–11412.

12. Gutowski, S., A. V. Smrcka, L. Nowak, D. G. Wu, M. Simon, and P. C. Sternweis. 1991. Antibodies to the α_q subfamily of guanine nucleotide-binding regulatory protein a subunits attenuate activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by hormones. *J. Biol. Chem.* 266:20519–20524.

13. Shenker, A., P. Goldsmith, C. G. Unson, and A. M. Spiegel. 1991. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G_q family. *J. Biol. Chem.* 266:9309–9313.

14. Taylor, S. J., H. A. Chae, S. G. Rhee, and J. H. Exton. 1991. Activation of the β_1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. *Nature (Lond.).* 350:516–518.

15. Smrcka, A. V., J. R. Hepler, K. O. Brown, and P. C. Sternweis. 1991. Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science (Wash. DC).* 251:804–807.

16. Tanner, F. C., G. Noll, C. M. Boulanger, and T. F. Lüscher. 1991. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries: Role of scavenger receptor and endothelium-derived nitric oxide. *Circulation.* 83:2012–2020.

17. Shimokawa, H., N. A. Flavahan, and P. M. Vanhoutte. 1990. Natural course of the impairment of endothelium-dependent relaxations in regenerating porcine endothelial cells: possible dysfunction of a pertussis toxin-sensitive G-protein. *Circ. Res.* 65:740–753.

18. Shimokawa, H., N. A. Flavahan, and P. M. Vanhoutte. 1991. Loss of endothelial pertussis toxin-sensitive G-protein function in atherosclerotic porcine coronary arteries. *Circulation.* 83:652–660.

19. Katada, T., O. Masayuki, and M. Ui. 1986. Two guanine nucleotid-binding proteins in rat brain serving as the specific substrate of islet-activating protein, pertussis toxin. *J. Biol. Chem.* 261:8182–8191.

20. Kanaho, Y., K. Takahashi, U. Tomita, T. Liri, T. Katada, M. Ui, and Y. Nozawa. 1992. A protein kinase C inhibitor, Staurosporine, activates phospholipase D via a pertussis toxin-sensitive GTP-binding protein in rabbit peritoneal neutrophils. *J. Biol. Chem.* 267:23554–23559.

21. Cerione, R. A., C. Staniszewski, J. L. Benovic, R. J. Lefkowitz, M. G. Caron, P. Giershik, R. Somers, A. M. Spiegel, J. Codina, and L. Birnbaumer. 1985. Specificity of the functional interactions of the β-adrenergic receptor and rhodopsin with guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J. Biol. Chem.* 260:1493–1500.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

23. Wilkie, T. M., P. A. Scherle, M. P. Strathmann, V. Z. Slepak, and M. I. Simon. 1991. Characterization of G-protein α subunits in the G_q class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc. Natl. Acad. Sci. USA.* 88:10049–10053.

24. Amatruda, T. T., D. A. Steele, V. Z. Slepak, and M. I. Simon. 1991. $G\alpha_{16}$, a G protein α subunit specifically expressed in hematopoietic cells. *Proc. Natl. Acad. Sci. USA.* 88:5587–5591.

25. Aragay, A. M., A. Katz, and M. I. Simon. 1992. The $G\alpha_q$ and $G\alpha_{11}$ proteins couple the thyrotropin-releasing hormone receptor to phospholipase C in GH_3 rat pituitary cells. *J. Biol. Chem.* 267:24983–24988.

26. Carlson, K. E., L. F. Brass, and D. R. Manning. 1989. Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than G_i in human platelets. *J. Biol. Chem.* 264:13298–13305.

27. Im, M. J., C. Gray, and A. J. Rim. 1992. Characterization of a phospholipase C activity regulated by the purified G_h in reconstitution systems. *J. Biol. Chem.* 267:8887–8894.

28. Liao, J. K., and C. J. Homcy. 1993. The release of endothelium-derived relaxing factor via α_2-adrenergic receptor activation is specifically mediated by G_{io2}. *J. Biol. Chem.* 268:19528–19533.

29. McKenzie, F. R., E. C. H. Kelly, C. G. Unson, A. M. Spiegel, and G. Milligan. 1988. Antibodies which recognize the C-terminus of the inhibitory guanine-nucleotide-binding protein (G_i) demonstrate that opioid peptides and foetal-calf serum stimulate the high-affinity GTPase activity of two separate pertussis-toxin substrates. *Biochem. J.* 249:653–659.

30. Simonds, W. F., P. K. Goldsmith, J. Codina, C. G. Unson, and A. M. Spiegel. 1989. G_{i2} mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes: In situ identification with Gα C-terminal antibodies. *Proc. Natl. Acad. Sci. USA.* 86:7809–7813.

31. McKenzie F. R., and G. Milligan. 1990. δ-opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein G_{i2}. *Biochem. J.* 267:391–398.

32. Logothetis, C. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. 1987. The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. *Nature (Lond.).* 325:321–326.

33. Camps, M., A. Carozzi, P. Schnabel, A. Scheer, P. J. Parker, and P. Giershik. 1992. Isozyme-selective stimulation of phospholipase C-β_2 by G protein $\beta\gamma$-subunits. *Nature (Lond.).* 360:684–686.

34. Katz, A., D. Wu, and M. I. Simon. 1992. Subunits $\beta\gamma$ of heterotrimeric G protein activate β_2 isoform of phospholipase C. *Nature (Lond.).* 360:686–689.

35. Huff, R. M., and E. J. Neer. 1986. Subunit interactions of native and ADP-ribosylated α_{39} and α_{41}, two guanine nucleotide-binding proteins from bovine cerebral cortex. *J. Biol. Chem.* 261:1105–1110.

36. Simon, B. C., L. D. Cunningham, and R. A. Cohen. 1990. Oxidized low-density lipoproteins cause contraction and inhibit endothelium-dependent relaxation in the pig coronary artery. *J. Clin. Invest.* 86:75–79.

37. Jacobs, M., F. Plane, and K. R. Bruckdorfer. 1990. Native and oxidized low-density lipoproteins have different inhibitory effects on endothelium-derived relaxing factor in the rabbit aorta. *Br. J. Pharmacol.* 100:21–26.

38. Flavahan, N. A. 1992. Atherosclerosis or lipoprotein-induced endothelial dysfunction: potential mechanisms underlying reduction in EDRF/ntric oxide activity. *Circulation.* 85:1927–1938.