Low molecular weight G-proteins of rho-family mediate relaxations to bradykinin in porcine coronary arteries¹

Toshiro SHIBANO², Paul M VANHOUTTE³

Center for Experimental Therapeutics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
³Department of Pharmacology, University of Hong Kong, Hong Kong, China

KEY WORDS bradykinin; endothelium; GTP-binding proteins; nitric oxide

ABSTRACT

AIM: To determine whether or not low molecular G-proteins are involved in the endothelium-dependent relaxations to bradykinin. METHODS: The effects of botulinum ADP-ribosyltransferase C3 were studied in porcine coronary arteries and endothelial cells. RESULTS: Incubation of membrane fractions isolated from endothelial cells with the enzyme and ³²P-NAD resulted in the ribosylation of the proteins with molecular weight of 24-25 kDa. Radio labelling of these proteins was suppressed in the presence of guanosine 5’-O-(3-thiotriphosphate) (GTP-γS), a hydrolysis-resistant analog of GTP. In the isolated arteries, ADP-ribosyltransferase C3 attenuated the relaxations to bradykinin during contractions with prostaglandin F₂α in the presence of tween 80 (non ionic detergent), but not in the absence of tween 80. CONCLUSION: Low molecular weight G-proteins of the Rho family contribute to the mechanism of relaxation induced by bradykinin.

INTRODUCTION

In the porcine coronary artery, 5-hydroxytryptamine and norepinephrine cause endothelium-dependent, pertussis toxin-sensitive relaxations by activating 5-HT₁D and α2-adrenoceptors, respectively, on endothelial cells¹⁻⁹. In the same preparation, bradykinin elicits an endothelium-dependent relaxation, mediated by B₂-kinin receptors, which consists of two components, one sensitive and one insensitive to inhibitors of nitric oxide synthase¹⁰⁻¹⁵. In coronary arteries covered with endothelial cells, that have regenerated after balloon denudation, responses mediated by Gi-proteins are reduced markedly, while that to bradykinin is preserved¹³,¹⁷. In contrast to 5-hydroxytryptamine and norepinephrine, the relaxations to bradykinin are relatively insensitive to pertussis toxin, which inhibits Gi-protein-coupled responses¹,³,⁴,¹⁸,¹⁹. Indeed, bradykinin receptors are coupled to both Gαi and Gαq families of G-proteins in endothelial cells, with the latter predominating¹,³,⁴,¹⁸,¹⁹. The release of nitric oxide evoked by bradykinin is not prevented by cholera toxin²². Endothelial cells express the Rho/Rho-kinase system²³⁻²⁵ which contributes to various cellular functions²⁶⁻³¹. In cultured endothelial cells, the activation of the phosphoinositol turnover evoked by bradykinin is inhibited by botulinum toxin (C2+C3 components), but not by pertussis toxin⁹,³²,³³. Botulinum ADP-ribosyl-transferase C3, produced by certain strains of clostridium botuli-
num type C and D, specifically inactivates the low molecular weight G-proteins RhoA/ Cdc42/Rac1, which are not ADP-ribosylated by either pertussis toxin or cholera toxin\cite{27,34,36}. The purpose of the present study was to examine the effects of botulinum ADP-ribosyltransferase C3 on the endothelium-dependent relaxations to bradykinin in porcine coronary arteries, to determine the role of low molecular weight G-proteins of the Rho family, in the response.

**MATERIALS AND METHODS**

Modification of GTP-binding proteins by ADP ribosyltransferase C3  Coronary arteries were removed from porcine hearts obtained from a slaughterhouse. The arteries were opened longitudinally and rinsed with Krebs-Ringer bicarbonate solution. Endothelial cells were harvested by scraping the intimal surface of the arteries with a scalpel blade\cite{7}. The endothelial cells were collected in control solution and washed by centrifugation. After sonication at 4 ºC for 30 min (Artek, 2×10^6 cpm) 1 mmol/L, and 10 mg proteins of crude ribosyltransferase C3 on the endothelium-dependent relaxations to bradykinin in porcine coronary arteries, to determine the role of low molecular weight G-proteins of the Rho family, in the response.

**Organ chamber studies** Left anterior descend-ing coronary arteries were rinsed in modified Krebs-Ringer bicarbonate solution [composition in mmol/L: NaCl 118.3; KCl 4.7; CaCl2 2.5; MgSO4 1.2; KH2PO4 1.2; NaHCO3 25; glucose 11.1; calcium-edetic acid 0.026 (control solution)], and then cut into rings (4-5 mm in length). The rings were suspended in organ chambers filled with control solution (aerated with 95 % O2 and 5 % CO2; pH 7.4, maintained at 37 ºC). Isometric force was measured by strain-gauge transducers (Statham UC2, Los Angeles, CA). The rings were stretched to the optimal point of their active length-tension curve (6 to 8 g). After one hour of equilibration, the rings were contracted with prostaglandin F20 (2 µmol/L), and responses to bradykinin (1×10^{-10}-3×10^{-8} mol/L) were obtained to confirm the presence of functional endothelium-dependent relaxations to the peptide. All experiments were performed in the presence of indomethacin (10 µmol/L) to prevent the formation of vasoactive prostanoids.

**Protocol of experiment 1 (effects of pertussis toxin on the relaxation to bradykinin)** Rings were incubated in the absence or presence of pertussis toxin (0.1 ng/L) for 90 min\cite{4,7}. Thereafter, rings were contracted with prostaglandin F20 (2 µmol/L), and responses to cumulatively increasing concentrations of bradykinin (1×10^{-10}-3×10^{-8} mol/L) were determined.

**Protocol of experiment 2 (effects of ADP-ribosyltransferase C3 in control solution)** The rings were divided into four groups. They were incubated in (a) control solution, (b) in the presence of ADP-ribosyltransferase C3 (0.5 ng/L); (c) in the presence of nitro-L-arginine (an inhibitor of nitric oxide synthase; 30 µmol/L\cite{37}), and (d) in the presence of ADP-ribosyltransferase C3 and nitro-L-arginine. After incubation for 90 min, responses to bradykinin [during contractions evoked by prostaglandin F20 (2 µmol/L)], were determined.

**Protocol of experiment 3 (effects of ADP-ribosyltransferase C3 in the presence of tween 80)** The rings were divided into five groups. In one group (a), the rings were incubated in control solution. In the other four groups (b, c, d, e), tween 80 (non-ionic detergent, 0.1 %) was added to the organ chambers to permeabilize plasma membranes to ADP-ribosyltransferase C3\cite{35,36}, (b) control solution, (c) in the presence of ADP-ribosyltransferase C3 (0.5 ng/L), (d) in the presence of nitro-L-arginine (30 µmol/L), and (e) in the presence of ADP-ribosyltransferase C3 and nitro-L-arginine. After incubation for 90 min, responses to
bradykinin (during contractions evoked by prostaglandin F$_{2\alpha}$), were determined.

**Materials** Adenosine 5'-triphosphate sodium salt (ATP), bovine serum albumin, bradykinin, β-nicotinamide adenine dinucleotide (NAD), indomethacin, pertussis toxin, monoclonal antibody against α-smooth muscle actin, and thymidine were obtained from Sigma Chemical Co (St Louis, MO); ADP-ribosyltransferase C3 (porcine brain) from Calbiochem (La Jolla, CA); nitro-L-arginine, tween 80 from Aldrich Chemical Co (Milwaukee, WIS); $^{125}$I-mouse Ig from Amersham (Arlington Heights, IL); reagents from polyacrylamide gel electrophoresis were from BioRad (Richmond, CA); and prostaglandin F$_{2\alpha}$ from Upjohn (Kalamazoo, MI); $^{32}$P-NAD, which was synthesized and provided by The Diabetes Center of Baylor College of Medicine, was a gift from Dr Juan Codina.

**Statistical analysis** Results in organ chamber studies are shown as mean±SEM, and $n$ refers to the number of animals from which coronary rings were obtained. Relaxations are expressed as percentage of the initial contractions to prostaglandin F$_{2\alpha}$. Statistical comparisons were performed by means of Student’s $t$-test for paired comparison and an analysis of variance (ANOVA) followed by Scheffe’s test when more than two groups were compared. $P$ values of less than 0.05 were considered to indicate statistically significant differences between groups.

**RESULTS**

**ADP-ribosylation of G-proteins** Western blotting using a monoclonal antibody against α-smooth muscle actin revealed bands around 42-45 kDa in the smooth muscle preparations, but no band was detected in membrane fractions obtained from endothelial cells (Fig 1). The assay of ADP-ribosylation of G-proteins following the incubation of the crude membrane fractions of endothelial cells with ADP-ribosyltransferase C3 and $^{32}$P-NAD on SDS-PAGE revealed a band around 24-25 kDa (Fig 2). In the absence of ADP-ribosyltransferase C3, the band was not detected. Treatment of the fractions with GTPγS (0.1 mol/L) reduced the intensity of the 24-25 kDa band (Fig 2).

**Organ chamber studies** There was no significant difference between groups in contractions to prostaglandin F$_{2\alpha}$. Bradykinin caused concentration-dependent, nitro-L-arginine-sensitive relaxations. Pertussis toxin did not affect the relaxations to bradykinin (Tab 1). ADP-ribosyltransferase C3 did not alter the resting tension of the rings (data not shown) and the relaxations to bradykinin (Fig 3). Nitro-L-arginine inhibited partially the relaxations to bradykinin, and the inhibition was not affected by ADP-ribosyltransferase C3 (Fig 3). Tween 80 did not alter resting tension or contractions to prostaglandin F$_{2\alpha}$ (control, 19.0±2.9 g; tween 80, 16.4±2.2 g; tween 80 and ADP-ribosyltransferase C3, 17.8±3.7 g; tween 80 and nitro-L-arginine, 20.0±4.4 g; tween 80, ADP-ribosyltransferase C3, and nitro-L-arginine, 17.0±2.6 g. $n$=6). Tween 80 did not affect the relaxations to bradykinin (Fig 4). The incubation of rings with ADP-ribosyltransferase C3 or nitro-L-arginine inhibited the relaxations to bradykinin in the presence of tween 80 (Fig 4). The inhibition of
Relaxations to bradykinin was significantly more pronounced with nitro-L-arginine than ADP-ribosyltransferase C3. The combined effect of ADP-ribosyltransferase C3 was the same as that of nitro-L-arginine alone.

**DISCUSSION**

Botulinum ADP-ribosyltransferase C3 selectively modifies low molecular (around 21-26 kDa) G-proteins of the Rho-family[23,25,27,33-35,38]. The molecular weight of the proteins (24-25 kDa) identified by SDS/autoradiography in the present studies were similar to those described in human umbilical vein endothelial cells[1]. The ADP-ribosylation of G-proteins catalysed by ADP-ribosyltransferase C3 is dependent on the concentration of Mg²⁺, and modified by guanine nucleotides[34]. GTPγS, a stable GTP analog, inhibits ADP-ribosylation in the presence of Mg²⁺, and enhances the reaction in the absence of divalent cations[34]. In the present study, incubation of membrane fractions with GTPγS in the presence of Mg²⁺ 2 mmol/L diminished the intensity of the band around 24-25 kDa. This indicates that the detected band is related to GTP-binding proteins in the native endothelial cells obtained from porcine coronary arteries.

The present study demonstrated that ADP-ribosylation of low molecular weight G-proteins inhibited the relaxations to bradykinin in the porcine coronary artery. The process was dependent on the activity of ADP-ribosyltransferase C3 and permeability of membranes by tween 80. Indeed, ADP-ribosyltransferase C3 did not affect the relaxations evoked by bradykinin in the absence of tween 80. There was no impairment of signal transduction at the concentration of tween 80 used. Although theoretically tween 80 may alter the responsiveness of coronary arteries, the detergent did not significantly affect contractions to prostaglandin F₂α and relaxations to bradykinin in the present study. ADP-ribosyltransferase C3 might affect the relaxations to bradykinin due to a direct action on the smooth muscle cells in coronary arteries. However, this is an unlikely explanation since the enzyme did not alter the endothelium-dependent, nitro-L-arginine-sensitive relaxations evoked by 5-hydroxytryptamine in the same preparation (data not shown). Endothelium-dependent relaxations elicited by bradykinin in the porcine coronary artery are mediated by two components which are either sensitive or insensitive to inhibitors of nitric oxide synthase[12-14]. In porcine aortic endothelial cells, activation of kinin B₂ receptors, mediating relaxation to

<table>
<thead>
<tr>
<th>Tab 1. Relaxations of porcine coronary arteries to bradykinin. Mean±SEM. *P&lt;0.05 vs control. **P&lt;0.05 vs Tween 80.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC₅₀/-lg mol·L⁻¹</strong></td>
</tr>
<tr>
<td>Experiment 1 (n=5)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Pertussis toxin 100 µg/L</td>
</tr>
<tr>
<td>Experiment 2 (n=4)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>ADP-ribosyltransferase C3 0.5 mg/L</td>
</tr>
<tr>
<td>Nitro-L-arginine 30 µmol/L</td>
</tr>
<tr>
<td>ADP-ribosyltransferase C3 plus nitro-L-arginine</td>
</tr>
<tr>
<td>Experiment 3 (n=6)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Tween 80 0.1 %</td>
</tr>
<tr>
<td>Tween 80 plus ADP-ribosyltransferase C3</td>
</tr>
<tr>
<td>Tween 80 plus nitro-L-arginine</td>
</tr>
<tr>
<td>Tween 80, ADP-ribosyltransferase C3, plus nitro-L-arginine</td>
</tr>
</tbody>
</table>

IC₅₀, effective concentration of bradykinin causing 50 % inhibition of the contractions to prostaglandin F₂α(2 µmol/L). Maximal relaxation: maximal relaxation in percentage of the contraction evoked by prostaglandin F₂α(2 µmol/L). ND, IC₅₀ values were not determined since the relaxations of some rings in the groups were less than 50 %.
bradykinin, release nitric oxide\cite{39,40}. The insensitive component to the inhibitors of nitric oxide synthase presumably is related to the release of endothelium-derived hyperpolarizing factor\cite{10,13,14}. Since ADP-ribosyltransferase C3 did not show further inhibition of the relaxations to bradykinin in the presence of nitro-L-arginine (NLA 30 µmol/L). n=4. Mean±SEM. bP<0.05 vs control.

Bradykinin stimulates phosphatidylinositol turnover and elevates inositoltriphosphate levels in porcine aortic endothelial cells\cite{32}. The stimulation of phospholipase C by bradykinin is not inhibited by pertussis toxin or cholera toxin\cite{42,43}. However, the stimulation is mediated by G-proteins since the responses are sensitive to GTP and analogs of the nucleotid\cite{42,43}. Low molecular GTP binding proteins (24 kDa) may regulate phospholipase C-coupled inositol lipid metabolism caused by bradykinin\cite{44}. Furthermore, botulinum toxin (C2 and C3 components) inhibits phosphoinositide turnover elicited by bradykinin in human umbilical vein endothelial cells\cite{33}. Thus, low molecular G-proteins are likely to be important mediators of the responses to bradykinin. The data presented here are consistent with a role for low molecular G-proteins in the release of nitric oxide by bradykinin, and the endothelium-dependent relaxation of the porcine coronary artery, evoked by the peptide.

ACKNOWLEDGMENTS The authors are grateful to the late Timothy SCOTT-BURDEN and to Dr Mark MILLAN for helpful discussion, to Mr Dewayne CONEY for technical assistance and to Ms Marie PALUMBO for editorial assistance.

REFERENCES

1 Flavahan NA, Shimokawa H, Vanhoutte PM. Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. J Physiol 1989; 408: 549-

Liao JK, Homcy CJ. The release of endothelium-derived relaxing factor via $\alpha_\text{1}$-adrenergic receptor activation is specifically mediated by Gi $\alpha_\text{i}$. J Biol Chem 1993; 268: 19528-33.


Marletta MA. Another activation switch for endothelial nitric oxide synthase: why does it have to be so complicated? Trends Biochem Sci 2001; 26: 519-21.


Voyno-Yasenetskaya TA, Tkachyuk VA, Cheknyoya EG,


