

Full-length article

Endothelium-derived hyperpolarizing factor mediated relaxations in pig coronary arteries do not involve Gi/o proteins^{1,2}Kwok Fu Jacobus NG^{3,4,5}, Susan Wai Sum LEUNG⁴, Ricky Ying Keung MAN⁴, Paul M VANHOUTTE⁴*Departments of³Anaesthesiology and⁴Pharmacology, The University of Hong Kong, Hong Kong, China***Key words**

endothelium-derived hyperpolarizing factor; pertussis toxin; Gi/o protein; pig coronary artery

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Abstract

Aim: Endothelium-dependent relaxations to certain neurohumoral substances are mediated by pertussis toxin-sensitive Gi/o protein. Our experiments were designed to determine the role, if any, of pertussis toxin-sensitive G-proteins in relaxations attributed to endothelium-derived hyperpolarizing factor (EDHF). **Methods:** Pig coronary arterial rings with endothelia were suspended in organ chambers filled with Krebs-Ringer bicarbonate solution maintained at 37 °C and continuously aerated with 95%O₂ and 5% CO₂. Isometric tension was measured during contractions to prostaglandin F_{2α} in the presence of indomethacin and N^ω-nitro-L-arginine methyl ester (L-NAME). **Results:** Thrombin, the thrombin receptor-activating peptide SFLLRN, bradykinin, substance P, and calcimycin produced dose-dependent relaxations. These relaxations were not inhibited by prior incubation with pertussis toxin, but were abolished upon the addition of charybdotoxin plus apamin. Relaxations to the α₂-adrenergic agonist UK14304 and those to serotonin were abolished in the presence of indomethacin and L-NAME. **Conclusion:** Unlike nitric oxide-mediated relaxations, EDHF-mediated relaxations of pig coronary arteries do not involve pertussis toxin-sensitive pathways and are Gi/o protein independent.

Introduction

Endothelium-dependent relaxations can be mediated through the release of nitric oxide (NO), prostacyclin, or endothelium-derived hyperpolarizing factor (EDHF)^[1]. Such relaxations can be initiated by the binding of different agonists to endothelial cell membrane receptors. The relaxations produced by certain (eg serotonin, thrombin, and α₂-adrenergic agonists) but not all (eg bradykinin) neurohumoral substances are mediated by pertussis toxin-sensitive Gi/o proteins^[2,3]. However, whether or not the EDHF-mediated component of these responses also depends on pertussis toxin-sensitive Gi/o proteins is not known.

EDHF-mediated responses include a number of different mechanisms that may exist in combination or in isolation^[1,4]. Upon activation of different endothelial receptors, there is an increase in the intracellular Ca²⁺ concentration and the opening of endothelial iK_{Ca} and sK_{Ca} channels. The combination of inhibitors of the iK_{Ca} and sK_{Ca} channels

apamin and charybdotoxin abolishes these responses^[5].

The hyperpolarization of endothelial cells is a result of the opening of these potassium channels. It then spreads to the underlying vascular smooth muscle cells by means of gap junctions, changes in interstitial K⁺ concentrations, release of epoxyeicosatrienoic acids (EET), lipoxygenase derivatives, hydrogen peroxide, endocannabinoids, or c-type natriuretic peptide (CNP). Theoretically, Gi/o proteins may be involved in the initial signal transduction upon endothelial receptor activation, or alternately, in the spread of hyperpolarization from the endothelium to the smooth muscle, particularly if this involves secretion of diffusible mediators.

The present experiments were designed to determine whether or not relaxations of pig coronary arteries that can be attributed to endothelium-dependent hyperpolarization involve Gi/o proteins and can be prevented or inhibited by pertussis toxin.

Materials and methods

Tissue preparation Pig hearts were collected from the local abattoir, where the animals were killed according to the regulations of the Food and Environmental Hygiene Department of the Hong Kong Special Administrative Region. The hearts were immediately rinsed several times and transported back to the laboratory in ice cold, oxygenated Krebs–Ringer bicarbonate solution (composition in mmol/L: NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25.0, and glucose 11.1; control solution). The right coronary arteries were dissected. The surrounding fat and connective tissue were removed, and the artery was cut into rings (with the endothelium; 4–5 mm in length). The rings were placed in jacketed organ chambers filled with 5 mL control solution. The solution was maintained at 37 °C and continuously aerated with 95% O₂/5% CO₂.

The rings were suspended vertically between 2 stainless-steel stirrups. One stirrup was anchored in the organ chamber, and the other was connected to a strain gauge for isometric tension recording. The rings were subjected to 5 g tension, which in preliminary experiments, was shown to be the optimal tension for rings of pig right coronaries obtained from the same source. Isometric tension was measured by means of force transducers (FT03; Grass Instruments, Quincy, MA, USA) coupled to an amplifier and a personal computer for data collection (PICO data logger; Pico Technology, Cambridge, UK).

The viability of each ring was determined by first contracting it 2–3 times with 30 mmol/L KCl followed by a single maximal contraction to 60 mmol/L KCl during which relaxation to bradykinin (1 µmol/L) was obtained. Rings that failed to produce a contraction greater than 4 g with 30 mmol/L KCl or relaxation greater than 40% with bradykinin were discarded. The rings were then washed repeatedly with the control solution.

Effect of pertussis toxin on EDHF-mediated relaxation After the re-establishment of a baseline tension of 5 g, the rings were incubated in control solution for 2 h in the presence or absence of pertussis toxin (400 ng/mL)^[6]. The rings were then contracted with prostaglandin F_{2α} (1–3 µmol/L) to a tension level approximating 30% of the maximal contraction produced by 60 mmol/L KCl. Once the contraction had stabilized, various agonists were added to produce relaxation. Indomethacin (10 µmol/L) was added to all rings 30 min prior to the initiation of contraction with prostaglandin F_{2α}. When other inhibitors, including N^ω-nitro-*L*-arginine methyl ester (*L*-NAME; 100 µmol/L), charybdotoxin (0.1 µmol/L), apamin (0.1 µmol/L), or ket-

anserin (a selective 5-HT₂ antagonist that inhibits the direct activating effect of serotonin on vascular smooth muscle; 10 µmol/L) were required, they were added at the same time as indomethacin. Pertussis toxin was allowed to remain in contact with the rings throughout the experiment.

Statistical analysis Data are presented as mean±SEM, with *n* representing the number of pig hearts used in the experiment. Curve fitting was performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) by fitting the data to the sigmoidal logistic equation:

$$R = \frac{R_{\max}}{1 + 10^{(\log EC_{50} - A) \cdot H}} \quad (1)$$

where *R* is the reduction in tone, *A* is the concentration of the agonist, *R*_{max} is the maximal relaxation, and *H* the slope function.

ANOVA was used for the comparison of curves among multiple treatment groups. A post-hoc comparison was performed by Newman-Keuls multiple comparison test where appropriate. Student's *t*-test for paired observations was used in the case of 2 group comparisons. *P*-values less than 0.05 were indicated statistically significant differences.

Drugs Indomethacin, ketanserin, *L*-NAME, charybdotoxin, apamin, prostaglandin F_{2α}, bradykinin, substance P, serotonin, UK14304, thrombin, and SFLLRN were purchased from Sigma (St Louis, MO, USA), and pertussis toxin was purchased from List Biological (Campbell, CA, USA). A stock solution of indomethacin was prepared in a 5 mmol/L sodium bicarbonate solution. A stock solution of pertussis toxin was prepared in 0.1 mol/L sodium phosphate (pH 7.0) and 0.5 mol/L NaCl. All other compounds were dissolved in deionized water. Concentrations are expressed as final molar concentrations in the bathing solution.

Results

Effect of pertussis toxin on EDHF-mediated relaxations by receptors agonists that may involve non-Gi/o coupling Bradykinin caused concentration-dependent relaxations which were reduced significantly by *L*-NAME (100 µmol/L). The presence of pertussis toxin (400 ng/mL) and *L*-NAME did not further inhibit relaxation. Charybdotoxin (0.1 µmol/L) and apamin (0.1 µmol/L) added to *L*-NAME nearly abolished the relaxations evoked by bradykinin (Figure 1A; Table 1). Similar results were obtained with substance P (Figure 1B; Table 1).

Effect of pertussis toxin on EDHF-mediated relaxation by receptor agonists that may involve Gi/o coupling Relaxations to thrombin (Figure 2A; Table 1) were attenu-

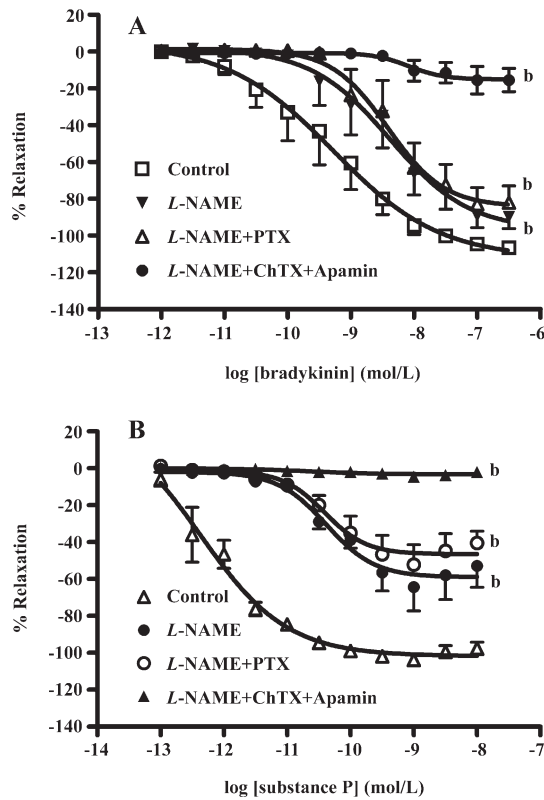


Figure 1. Cumulative concentration-response curves for (A) bradykinin [BK (1 pmol/L–0.3 μmol/L)] (*n*=7) and (B) substance P [SP (0.1 pmol/L–10 nmol/L)] (*n*=8) in pig coronary arterial rings with endothelium contracted with prostaglandin F_{2α} [PGF_{2α} (1–3 μmol/L)]. The rings were incubated with indomethacin (10 μmol/L, 30 min), with or without L-NAME (100 μmol/L, 30 min) and/or pertussis toxin [PTX (400 ng/mL, 2 h)], charybdotoxin [ChTX (0.1 μmol/L, 30 min)] and apamin (0.1 μmol/L, 30 min). Each point represents percentage relaxation as mean±SEM. ^b*P*<0.05 compared with control.

ated by L-NAME (100 μmol/L) and abolished by charybdotoxin (0.1 μmol/L) and apamin (0.1 μmol/L) on top of

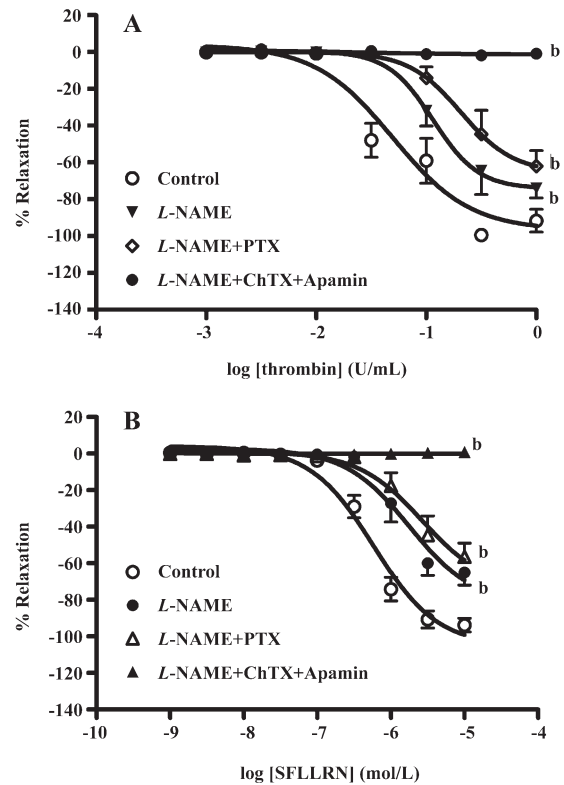


Figure 2. Cumulative concentration-response curves for (A) thrombin (0.001–1 U/mL) (*n*=9) and (B) SFLLRN (1 nmol/L–10 μmol/L) (*n*=7) in pig coronary arterial rings with endothelium contracted with prostaglandin F_{2α} [PGF_{2α} (1–3 μmol/L)]. The rings were incubated with indomethacin (10 μmol/L, 30 min), with or without L-NAME (100 μmol/L, 30 min) and/or pertussis toxin [PTX (400 ng/mL, 2 h)], charybdotoxin [ChTX (0.1 μmol/L, 30 min)] and apamin (0.1 μmol/L, 30 min). Each point represents percentage relaxation as mean±SEM. ^b*P*<0.05 compared with control.

L-NAME. Incubation with pertussis toxin (400 ng/mL) did not significantly affect the L-NAME-resistant component of relaxation. A similar pattern of inhibition was observed for SFLLRN (Figure 2B; Table 1) with L-NAME, charybd-

Table 1. Effects of pertussis toxin [PTX (400 ng/mL, 2 h)] on maximal relaxation and EC₅₀ to bradykinin, substance P (SP), thrombin, SFLLRN and calcimycin in pig coronary arterial rings with endothelium contracted with prostaglandin F_{2α} [PGF_{2α} (1–3 μmol/L)]. All arterial rings were treated with indomethacin (10 μmol/L) and some with L-NAME (100 μmol/L). *n*=5–9. Data are mean (95% CI) for ED₅₀ and mean±SEM. for maximal relaxation. ^b*P*<0.05 vs control.

Agonist	EC ₅₀ (mol/L or U·mL ⁻¹)			Maximal relaxation (%)		
	Control	L-NAME	L-NAME+PTX	Control	L-NAME	L-NAME+PTX
Bradykinin	5.3 (1.7–16.1)×10 ⁻¹⁰	4.1 (1.4–12.5)×10 ^{-9b}	3.8 (1.8–8.3)×10 ^{-9b}	112±10	97±12	84±8 ^b
SP	4.2 (0.6–32.7)×10 ⁻¹³	4.1 (2.3–7.5)×10 ^{-11b}	3.9 (2.2–7.0)×10 ^{-11b}	102±3	59±3 ^b	47±2 ^b
Thrombin	5.0 (2.9–8.6)×10 ⁻²	1.2 (0.8–1.6)×10 ^{-1b}	2.1 (1.1–3.9)×10 ^{-1b}	97±8	74±6 ^b	65±9 ^b
SFLLRN	5.9 (3.1–11.0)×10 ⁻⁷	1.8 (0.7–4.2)×10 ⁻⁶	2.7 (1.4–5.2)×10 ^{-6b}	ND	ND	ND
calcimycin	1.4 (0.6–3.1)×10 ⁻⁸	6.5 (2.8–15.1)×10 ^{-8b}	5.5 (2.0–15.0)×10 ^{-8b}	102±5	107±8	101±9

ND=not determined.

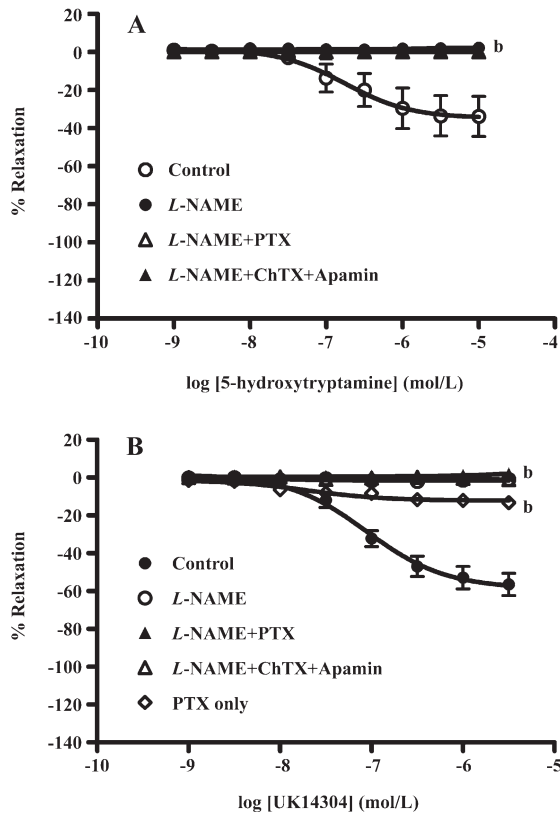


Figure 3. Cumulative concentration-response curves for (A) serotonin [5-HT (1 nmol/L–10 μ mol/L)] in the presence of ketanserin (10 μ mol/L) ($n=7$); and (B) 5-bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine [UK14304 (1 nmol/L–3 μ mol/L)] ($n=4-8$) in pig coronary arterial rings with endothelium contracted with prostaglandin $F_{2\alpha}$ [PGF $_{2\alpha}$ (1–3 μ mol/L)]. The rings were incubated with indomethacin (10 μ mol/L, 30 min), with or without *L*-NAME (100 μ mol/L, 30 min) and/or pertussis toxin [PTX (400 ng/mL, 2 h)], charybdotoxin [ChTX (0.1 μ mol/L, 30 min)] and apamin (0.1 μ mol/L, 30 min). Each point represents percentage relaxation as mean \pm SEM. ^b $P<0.05$ compared with control.

dotoxin, and apamin. Pertussis toxin did not significantly affect *L*-NAME-resistant relaxation to SFLLRN. No relaxation was produced by serotonin or UK14304 in the presence of *L*-NAME (100 μ mol/L; Figure 3A, 3B). NO-dependent relaxation to UK14304 was significantly inhibited by pertussis toxin (Figure 3B).

Effect of pertussis toxin on EDHF-mediated relaxation independent of endothelial surface receptors The relaxation to calcimycin (Figure 4; Table 1) was attenuated by *L*-NAME (100 μ mol/L) and was nearly abolished with charybdotoxin (0.1 μ mol/L) and apamin (0.1 μ mol/L) on top of *L*-NAME. Incubation with pertussis toxin (400 ng/mL) again did not significantly affect the *L*-NAME-resistant component of the relaxation.

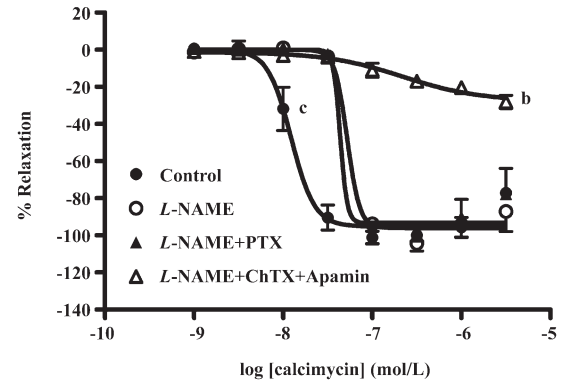


Figure 4. Cumulative concentration-response curves for calcimycin (1 nmol/L–3 μ mol/L)] ($n=5$) in pig coronary arterial rings with endothelium contracted with prostaglandin $F_{2\alpha}$ [PGF $_{2\alpha}$ (1–3 μ mol/L)]. The rings were incubated with indomethacin (10 μ mol/L, 30 min), with or without *L*-NAME (100 μ mol/L, 30 min) and/or pertussis toxin [PTX (400 ng/mL, 2 h)], charybdotoxin [ChTX (0.1 μ mol/L, 30 min)] and apamin (0.1 μ mol/L, 30 min). Each point represents percentage relaxation as mean \pm SEM. ^b $P<0.05$, ^c $P<0.05$ compared with control for *L*-NAME and *L*-NAME plus pertussis toxin at 10 and 30 nmol/L concentrations.

Discussion

This study investigates the dependence, if any, of EDHF-mediated relaxations of pig coronary arteries on pertussis toxin-sensitive Gi/o proteins based on previous findings that certain NO-mediated responses are Gi/o protein dependent^[2,3]. EDHF-mediated responses were obtained after the inhibition of the NO pathway with *L*-NAME and the prostacyclin pathway with indomethacin in arteries with endothelia. These responses were abolished in the presence of charybdotoxin and apamin, confirming that these are EDHF-mediated responses^[1].

Hyperpolarization of vascular smooth muscles in EDHF-mediated responses may follow that of the endothelial cells via gap junctions, changes in interstitial K⁺ concentrations, release of EET, lipoxygenase derivatives, CNP, endocannabinoids, or hydrogen peroxide^[1,4,7-9]. As the final effector mechanisms are so varied, there are likely to be multiple pathways linking the initial activation of endothelial receptors to these numerous mechanisms. As Gi/o proteins may be involved in both transduction and effector mechanisms, it is intuitively quite possible that they are involved in at least part of the entire EDHF-mediated response. Pertussis toxin inhibits EDHF-mediated responses to carbachol, the endocannabinoid anandamide, and Δ^9 -tetrahydrocannabinol, as well as calcimycin in small mesenteric arteries of rats^[8,10]. It also inhibits relaxation by CNP, a putative EDHF, again in the rat mesenteric artery^[7]. However, this was found not to be the case

in pig coronary arteries in the present study. This may be explained by the different agonists used to induce EDHF responses in the present experiment or by species differences. Thus, pertussis toxin does not cause inhibition of the EDHF-mediated response in the rabbit carotid artery^[11].

Previous studies on regenerated endothelia after denudation by balloon angioplasty also support the notion that EDHF-mediated responses are independent of Gi/o proteins in pig coronary arteries. The dysfunction of the Gi/o protein pathway^[12,13] is of major importance in the impairment of endothelium-dependent relaxations in coronaries with regenerated endothelium^[3,14], however, these arteries frequently exhibit a compensatory increase in the EDHF-mediated response^[15]. Similar observations have also been made in hyperlipidemia and atherosclerosis, as Gi/o protein pathway dysfunction has been observed under these conditions^[16-18]. Again, a compensatory increase in EDHF-mediated relaxation is observed^[19], despite Gi/o protein pathway dysfunction. Lastly, Gi/o protein expression is low in small coronary arteries^[20] and preparations known to exhibit prominent EDHF activity^[21-23].

The findings of the present study are in line with these observations. In pig coronary arteries, all of the EDHF-mediated responses studied appear to be independent of Gi/o protein pathways as they were unaffected by pertussis toxin. This was the case for agonists, such as bradykinin and substance P that elicit a prominent EDHF-component during endothelium-dependent relaxations in this preparation. Bradykinin induces relaxation by activating B₂ kinin receptors, and substance P induces relaxation via the NK₁ receptor^[24]. Both receptors are coupled to the Gq protein and involve the phospholipase C, inositol 1,4,5-triphosphate, and diacylglycerol system as effector systems^[25]. As pertussis toxin selectively ADP-ribosylates the α -subunits of Gi/o proteins, it is plausible that the effects of bradykinin and substance P are not affected by pertussis toxin. The absence of the effect of pertussis toxin on bradykinin-induced relaxation has been reported previously^[2].

Thrombin induces endothelium-dependent relaxations by activating protease-activated receptor (PAR)₁, which is coupled to Gi/o proteins^[2,26]. In the present experiment, however, EDHF-mediated responses to PAR activation remained intact after incubation with pertussis toxin. This was the case with both the natural agonist of PAR, thrombin, as well as with the PAR₁-selective tethered ligand thrombin receptor activating peptide, SFLLRN. Both produce endothelium-dependent relaxations, but differences exist between the 2 PAR agonists. Thus, in pig coronary arteries, the non-NO component of endothelium-dependent relaxations to thrombin, but not that to SFLLRN, are inhibited by L-type voltage-operated calcium channel blockers,

such as nifedipine^[27]. In the present study, a similar EDHF-mediated relaxation was obtained with both thrombin and SFLLRN. This relaxation was not affected by pertussis toxin in both cases. This then prompts the conclusion that the Gi/o protein pathway is not involved, at least in the EDHF-mediated relaxation following PAR₁ activation. This finding is of particular importance because of the role of thrombin as a key enzyme in blood coagulation, platelet activation, and inflammation^[28]. Thrombin is likely to be present in the vicinity of the endothelium during critical events, such as thrombus formation and plaque rupture. The present study confirms that thrombin maintains its ability to induce EDHF-mediated relaxations independent of Gi/o proteins. This may allow thrombin to produce a more favorable vascular tone during such critical events.

This study also investigates 2 agonists with known dependence on the Gi/o protein pathway to produce endothelium-dependent relaxations, namely serotonin^[14] and the α 2-adrenergic agonist UK14304. However, in the present study both serotonin and UK14304 failed to produce EDHF-mediated relaxations. Therefore, the contribution of the Gi/o protein pathway to EDHF responses induced by these 2 agonists cannot be assessed in pig coronary arteries.

In addition to being coupled to endothelial surface receptors, Gi/o proteins, and thus pertussis toxin-sensitive pathways, may also be involved in the mechanisms leading to hyperpolarization of vascular smooth muscle cells. For instance, the relaxations induced by CNP, which is a possible candidate for EDHF, may involve the action of CNP on a Gi/o-coupled smooth muscle K⁺ channel^[29]. This and other similar mechanisms are investigated in this study using calcimycin to directly increase endothelial intracellular Ca²⁺ concentrations, bypassing the effects of G-protein coupling to endothelial surface receptors. Again, no pertussis toxin-dependent effect was observed.

The major conclusion derived from the present experiments is that EDHF-mediated relaxations of pig coronary arteries are not dependent on pertussis toxin-sensitive Gi/o protein pathways. This independence of EDHF-mediated relaxations on Gi/o proteins is universal across a variety of agonists and probably a full range of different mechanisms of EDHF generation. This shows that in large coronary arteries, which are the major sites affected by atherosclerosis and plaque formation, and also the principal site of coronary interventions, NO- and EDHF-mediated endothelium-dependent relaxations are distinct mechanisms. These mechanisms not only differ in terms of the mediator(s) involved, but also have distinct signal transduction systems. These 2 pathways can therefore serve complementary roles, particularly under diseased conditions, such as the dysfunction caused by regeneration or atherosclerosis.

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Author contribution

Kwok Fu Jacobus NG, Ricky Ying Keung MAN, and Paul M VANHOUTTE designed research; Kwok Fu Jacobus NG and Susan Wai Sum LEUNG performed research; Kwok Fu Jacobus NG, Susan Wai Sum LEUNG, Ricky Ying Keung MAN, and Paul M VANHOUTTE analyzed data; Kwok Fu Jacobus NG, Ricky Ying Keung MAN, and Paul M VANHOUTTE wrote the paper.

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