Role of pertussis toxin-sensitive G protein in metabolic vasodilation of coronary microcirculation

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We have previously demonstrated that pertussis toxin (PTX)-sensitive G protein (GPTX) plays a major role in coronary microvascular vasomotion during hypoperfusion. We aimed to elucidate the role of GPTX during increasing metabolic demand. In 18 mongrel dogs, coronary arteriolar diameters were measured by fluorescence microangiography using a floating objective. Myocardial oxygen consumption (MV˙O2) were measured by fluorescence microangiography using a demand. In 18 mongrel dogs, coronary arteriolar diameters were increased by rapid left atrial pacing. In six dogs, PTX (300 ng/ml) was superfused onto the heart surface for 2 h to locally block GPTX. In eight dogs, the vehicle (Krebs solution) was superfused in the same way. Before and after each treatment, the diameters were measured during control (130 beats/min) and rapid pacing (260 beats/min) in each group. Metabolic stimulation before and after the vehicle treatment, the diameters were measured during control (130 beats/min) and rapid pacing (260 beats/min) in each group. Metabolic stimulation before and after the vehicle treatment caused 8.6 ± 1.8 and 16.1 ± 3.6% dilation of coronary arterioles <100 μm in diameter (57 ± 8 μm at control, n = 10), respectively. PTX treatment clearly abolished the dilation of arterioles (12.8 ± 2.5% before and 0.9 ± 1.6% after the treatment, P < 0.001 vs. vehicle; 66 ± 8 μm at control, n = 11) in response to metabolic stimulation. The increases in MVO2 and coronary flow velocity were comparable between the vehicle and PTX groups. In four dogs, 8-phenyltheophylline (10 μM, superfusion for 30 min) did not affect the metabolic dilation of arterioles (15.3 ± 2.0% before and 16.4 ± 3.8% after treatment; 84.3 ± 11.0 μm at control, n = 8). Thus we conclude that GPTX plays a major role in regulating the coronary microvascular tone during active hyperemia, and adenosine does not contribute to metabolic vasodilation via GPTX activation.

coronary circulation; active hyperemia; guanine nucleotide regulatory protein

GTP-BINDING REGULATORY PROTEINS (G proteins) play central roles in transducing various biological signals from the outside to the inside of the cell. Many endogenous substances that bind to their specific receptors on the surface of the cell membrane activate specific G proteins and modulate the activity of effectors such as ion channels and enzymes.

When the oxygen supply to the myocardium is suppressed, coronary arteries dilate via the activation of ATP-sensitive K+ (KATP) channels, as observed in hypoxemia (8), hypoperfusion (7, 30, 40), and reactive hyperemia (3, 25). In addition to these observations, we have previously demonstrated that pertussis toxin (PTX)-sensitive G protein (GPTX) plays a crucial role in the coronary microvascular regulation of vasomotor tone during hypoperfusion in vivo (32).

However, coronary perfusion is predominantly regulated by the myocardial metabolic state in physiological conditions. The mechanisms that govern the vascular responses to metabolic changes are still unclear and controversial. Although the mediators of the local metabolic coronary control have not yet been identified, many substances (such as adenosine, prostacyclin, nitric oxide, H+., etc.) have been proposed as candidates, and the contribution of KATP channels has also been suggested. Many reports (4, 13, 23, 26, 43) have suggested that KATP channels and/or nitric oxide may be involved in metabolic coronary vasodilation. For example, Katsuda et al. (27) and Duncker et al. (9) suggested that KATP channels are involved in metabolic coronary vasodilation in dogs. On the other hand, Jones et al. (23) and Quyyumi et al. (43) showed that an inhibitor of nitric oxide synthase significantly reduced the metabolic coronary vasodilation induced by rapid pacing in dogs and humans. Embrey et al. (13) showed that metabolic coronary vasodilation is mediated through a nitric oxide-dependent mechanism. Thus most reports have supported the contribution of KATP channels and/or the nitric oxide pathway in metabolic coronary vasodilation.

We (31) recently reported that direct activation of GPTX causes vasodilation via the L-arginine-nitric oxide pathway and via hyperpolarization by KATP channel activation in the coronary microcirculation in vivo. These findings suggest that GPTX might also contribute...
to coronary vasodilation via nitric oxide or K\textsubscript{ATP} channel activation not only during hypoperfusion but also during increasing metabolic demand. Accordingly, the purpose of the present study was to elucidate the role of G\textsubscript{PTX} in active hyperemia of the coronary microcirculation.

**MATERIALS AND METHODS**

**General Preparation**

Eighteen small mongrel dogs of both sexes, weighing 6.3–11.2 kg, were premedicated with ketamine (10 mg/kg im) and then anesthetized with an intravenous injection of α-chloralose (60 mg/kg, Wako Chemicals, Osaka, Japan). Additional doses, if necessary, were given to maintain anesthesia. The animals were intubated and mechanically ventilated (model NSH-34RH, Harvard Apparatus, South Natick, MA) at an end-expiratory pressure of 3–5 cmH\textsubscript{2}O. Metabolic acidosis during the experiments was prevented by intravenous infusion of sodium bicarbonate. Arterial blood gases were maintained within the physiological range by adjusting the rate and volume of a ventilator and/or by using oxygen-enriched air. Body temperature was maintained at 37–38°C by means of a homeothermic blanket system. A catheter was introduced into the right external jugular vein for the infusion of drugs and fluid. Aortic pressure was measured at the aortic root with a catheter passed through the right carotid artery and connected to a strain-gauge pressure transducer (model MPU 0.5, Toyo Sokki, Tokyo, Japan). A thoracotomy was performed in the fifth left intercostal space, and the heart was suspended in a pericardial cradle. A snare was placed around the descending thoracic aorta, and a balloon catheter was placed in the inferior vena cava through a right femoral vein for the control of aortic pressure. The heart rate was controlled by left atrial pacing after sinus node suppression with a local injection of 10% buffered formaldehyde (0.3–0.5 ml).

To calculate the relative change of myocardial oxygen consumption (MVO\textsubscript{2}), we measured the oxygen saturation of paired blood samples from the aorta (SAO\textsubscript{2}) and the coronary sinus (ScsO\textsubscript{2}). Macho et al. (34) previously reported that the dilation of epicardial large coronary arteries was minimal during metabolic stimulation by rapid pacing in conscious dogs. In that report, epicardial conduit arteries (3.95 ± 0.19 mm in control diameter) dilated by 0.07 ± 0.01 mm when MVO\textsubscript{2} increased by 34%. Therefore, it is possible to substitute mm for control diameter) dilated by 0.07

$$k = 0.07$$

where $k$ is a constant that equals the mean coronary flow velocity during rapid pacing divided by the mean coronary flow velocity during control pacing. Mean and phasic blood flow velocities of the left circumflex coronary artery were continuously measured using a 20-MHz small succion-cupped Doppler probe connected to a pulsed Doppler flowmeter (model 100, Triton Technology, San Diego, CA). Blood sampling and mean velocity measurements were done just after the image acquisition of the microvessels.

A catheter was introduced into the left atrium via the left atrial appendage for the injection of fluorescein isothiocyanate dextran (molecular weight: 154,200; Sigma Chemical, St. Louis, MO). The exposed cardiac surface was kept moist during the experiments by a continuous drip of warm physiological solution containing (in mM) 118.2 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 25.0 NaHCO\textsubscript{3}, 0.026 calcium disodium EDTA, and 5.5 glucose maintained at 37°C and a pH of 7.40. To reduce excessive cardiac movement, two 24-gauge stainless steel needles were inserted horizontally (5–7 mm apart) into the midmyocardium of the left ventricle. Both ends of each needle were fixed to a needle holder held with coil springs. This apparatus allowed the heart to move perpendicularly but limited excessive horizontal movement so that the area of interest was held in the microscopic field of view. Aortic pressure and mean and phasic coronary blood flow velocities were simultaneously recorded on a rectigraph (type 8K 12-1s-ME, San-Ei Sokki, Tokyo, Japan).

**Microscope System**

The complete details of the microscope system equipped with a floating objective have been described elsewhere (1, 2). Briefly, a floating objective consists of a pair of convex lenses aligned on a common optical axis; this apparatus transmits the real image of the epimyocardium of the beating heart to a fixed position without any change in magnification. The convex lens facing the heart can move in unison with the cardiac motion without touching the cardiac surface. The real image of an object on the front focus of this lens is transmitted to the back focus of the second convex lens with parallel light. Consequently, changes in distance between these two convex lenses do not affect the position and magnification of the transmitted real image. This transmitted real image is then observed with a standard microscope. The convex lens facing the heart is mounted in a thin aluminum tube (floating lens) to reduce its total weight (16 g). The floating lens is supported by six low-resistance ball bearings and is suspended by a weight-adjusting coil spring. A 20-gauge stainless steel needle was inserted into the midmyocardium using a micromanipulator. The needle was attached to a needle holder, which allowed the tip of the needle to move up and down in unison with the cardiac motion. To adjust the focal distance between the floating lens and the heart, the floating lens was lifted just above the surface of the heart by a lifter that was connected to a needle holder. The height of the lifter was controlled with an oil pressure micromanipulator.

**Measurement of Microvascular Diameters**

For the measurement of coronary microvascular diameters, epi-illuminated fluorescence microangiography was performed. Fluorescein isothiocyanate dye (4–5 mg, 20 mg/ml in saline) was injected as a bolus into the left atrium. The left ventricular surface was epi-illuminated using a mercury lamp (model HBO-100EW/2, Nikon, Tokyo, Japan). The maximal wavelength of the illumination light was 495 nm, obtained with a B2 excitation filter (Nikon). The emitted light was passed through a 510-nm filter. Microvascular fluorescent images were recorded at 200 frames/second using a high-speed video system (MHS-200, Nac, Tokyo, Japan) connected to an image intensifier (C3100, Hamamatsu Photonics, Hamamatsu, Japan). In the end-diastolic phase, arteriolar diameters were measured on a high-resolution monitor screen (model C 1846-01, Hamamatsu Photonics) using a video manipulator (model C2117, Hamamatsu Photonics). To define the end-diastolic phase, the electrocardiogram wave was inserted on the video screen using a wave inserter.
was made. Thereafter, 8-phenyltheophylline (10 \text{mM}) was superfused topically until the end of the experiment. Thirty minutes after the onset of 8-phenyltheophylline superfusion, adenosine was applied again, and the measurements of the hemodynamics and diameter were repeated.

**Experimental Protocol 1**

Experiments were performed ~30 min after the surgical preparation and instrumentation, when all monitored variables had become stable.

Eighteen dogs were divided into three groups: the PTX group (n = 6), the vehicle group (n = 8), and the 8-phenyltheophylline group (n = 4). In the PTX group, the control coronary microvascular diameters, hemodynamic variables, and blood gas data were measured 5 min after the onset of left atrial pacing at ~130 beats/min (control pacing) and then after the pacing rate was increased to ~260 beats/min (rapid pacing). Five minutes after the onset of rapid pacing, the coronary microvascular diameters, hemodynamic variables, and blood gas data were measured again. After the above measurements were taken, the pacing rate was returned to the basal value (140 ± 8 beats/min), and the PTX (300 ng/ml) superfusion was started and continued for 2 h to locally block G_{PTX}. After the pretreatment with PTX, the coronary microvascular diameters, hemodynamic variables, and blood gas data were measured again during control pacing (130 beats/min) and rapid pacing (260 beats/min). In the vehicle group, the procedures employed were similar to those of the PTX group except for the superfusion of Krebs solution instead of PTX. In the 8-phenyltheophylline group, the procedures employed were similar to those of the PTX group except for the superfusion of 8-phenyltheophylline (10 \text{mM}) instead of PTX onto the heart surface for 30 min after the control measurement. Finally, in each group, sodium nitroprusside (100 \text{ng/ml}) was superfused onto the heart surface to evaluate the non-specific effect of PTX or 8-phenyltheophylline on the vasodilation reserve. In all protocols, additional doses of \alpha-chloralose (~10 \text{mg/kg}) were usually injected every half hour to maintain an adequate anesthesia level during the experiments. Blood pressure was continuously monitored, and blood samples for analyses of blood gases and blood pH were obtained at the start and end of each experiment.

In four dogs, the effect of 8-phenyltheophylline on adenosine-induced vasodilation was evaluated. We topically superfused 10^{-8}-10^{-5} \text{M} adenosine. Ten minutes after the onset of superfusion of each concentration of the drugs, hemodynamic variables and coronary microvascular diameters were measured. Thirty minutes after the adenosine superfusion was discontinued, a second control measurement was made. Thereafter, 8-phenyltheophylline (10 \text{mM}) was superfused topically until the end of the experiment. Thirty minutes after the onset of 8-phenyltheophylline superfusion, adenosine was applied again, and the measurements of the hemodynamics and diameter were repeated.

**Experimental Protocol 2**

To examine the direct effect of PTX on the myocardium, we measured the developed force of the cardiac muscle elicited by electrical stimulation using a modified silicon semiconductor strain gauge (model AE 801, SensoNor, Horten, Norway), as previously described (36). Mongrel dogs of both sexes were anesthetized with \alpha-chloralose, and the hearts were rapidly removed. After the heart was arrested, the trabeculae (n = 5; length: 1.52 ± 0.14 mm, width: 450 ± 82 \text{mum}, and thickness: 240 ± 58 \text{mum}) were dissected from five right ventricles and mounted horizontally between a force transducer and a micromanipulator in a perfusion bath located on the stage of an inverted microscope (Nikon). The trabeculae were stimulated at 0.5 Hz through parallel platinum electrodes in the bath with 5-ms pulses 50% above threshold and superfused with the physiological solution described in General Preparation. The force signal was displayed on an oscilloscope, recorded with a chart recorder, and stored digitally (16 bit, 10 kHz) on a data recorder (RD-130TE Dat Data Recorder, TEAC, Tokyo, Japan) for later analysis. The twitch force of the trabeculae reached a steady level within 60 min after the dissection, and the steady state lasted for at least 4 h. Before the experiment was started, the variation of the developed force during the steady state was <5%. The muscle lengths of all trabeculae were set at a level at which the resting force was 5% of the maximal force development. The temperature was set at 28.0 ± 0.1°C.

The force development at 0.5 and 1.0 Hz stimulation was recorded during the steady state when the superfusion was with the physiological solution. The trabeculae were then superfused with the physiological solution containing 300 ng/ml PTX. After the trabeculae were superfused with PTX for 1 h, the force development at 0.5 and 1.0 Hz stimulation was recorded again. To assess the direct effect of PTX on myocardial contractility, we calculated the change in force over time (dF/dt) from the recordings of the force development.

**Preparation of Drugs**

Lyophilized PTX (100 \mu g; Seikagaku, Tokyo, Japan) was dissolved with 10 ml of distilled water and stored at 4°C. The PTX solution was freshly diluted with Krebs solution to 300 ng/ml on the day of each experiment. Sodium nitroprusside (Wako Chemicals) was freshly dissolved with Krebs solution. 8-Phenyltheophylline (Sigma Chemical) was dissolved in a minimal amount of 5 N NaOH and methanol and then diluted with Krebs solution to the required final concentration (10 \mu M). Adenosine (Sigma Chemical) was dissolved in saline.

**Administration of Drugs**

As mentioned above, Krebs solution (at 37°C) was continuously superfused onto the observed area with a syringe pump (STC 521, Terumo, Tokyo, Japan) throughout the experiment at the rate of 60 ml/h, unless otherwise stated. When PTX was applied, the superfusion of Krebs solution was stopped, and PTX (300 ng/ml) was superfused onto the heart surface with a syringe pump (STC 521, Terumo) at a rate of 10 ml/h. When 8-phenyltheophylline was applied, the superfusion of Krebs solution was stopped, and 8-phenyltheophylline (10 \mu M) was superfused onto the heart surface with a syringe pump at a rate of 10 ml/h. The entire superfusion line was continuously warmed by a warm-water circuit using a thermostat-controlled water bath to keep the superfusate temperature at 37°C on the heart surface. For the superfu-
paired samples was applied. When the differences were accepted as significant. PTX-treated groups (solid bars). vehicle- (Krebs solution) (open bars) and
and mean coronary flow velocity between consumption (MV˙O₂, myocardial oxygen consumption; PTX, pertussis toxin. *P < 0.05 vs. control.

**RESULTS**

**Arterial Blood Gases, pH, and Systemic Hemodynamics**

Table 1 shows the hemodynamics and blood gas data collected during the experiments. Arterial blood gases and pH were maintained within physiological ranges throughout the experiments. Systolic, diastolic, and mean aortic pressures were near control levels throughout the experiments in all protocols. There were no statistical differences in the values of control oxygen saturation of the coronary sinus between the two groups. There were no significant differences in heart rate and mean aortic pressure at control and rapid pacing between the vehicle and PTX groups. Rapid pacing caused a significant decrease in the oxygen saturation of the coronary sinus and caused a significant increase in MV˙O₂.

**Effect of Metabolic Stimulation on MV˙O₂ and Coronary Flow Velocity**

As shown in Figure 1, rapid atrial pacing similarly increased MV˙O₂ and mean coronary flow velocity before

**Table 1. Arterial blood gases, pH, and systemic hemodynamics**

<table>
<thead>
<tr>
<th></th>
<th>Before Treatment</th>
<th>After Treatment</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle Group</td>
<td>PTX Group</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Rapid pacing</td>
</tr>
<tr>
<td>pH</td>
<td>7.39 ± 0.01</td>
<td>7.38 ± 0.01</td>
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<tr>
<td>PVo₂, mmHg</td>
<td>98 ± 4</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>PCO₂, mmHg</td>
<td>32 ± 1</td>
<td>35 ± 1</td>
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<tr>
<td>HR, beats/min</td>
<td>126 ± 5</td>
<td>259 ± 12</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>119 ± 4</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>DAP, mmHg</td>
<td>87 ± 4</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>99 ± 4</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>SVO₂, %</td>
<td>97.3 ± 0.4</td>
<td>97.2 ± 0.3</td>
</tr>
<tr>
<td>MVO₂, % of control</td>
<td>45.8 ± 2.7</td>
<td>41.8 ± 3.0*</td>
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Values are means ± SE; n = 8 hearts in the vehicle (Krebs solution) group and n = 6 hearts in the PTX group. HR, heart rate; SAP, systolic aortic pressure; DAP, diastolic aortic pressure; MAP, mean aortic pressure; SVO₂, oxygen saturation of the coronary sinus; MVO₂, myocardial oxygen consumption; PTX, pertussis toxin. *P < 0.05 vs. control.

**Data Analysis**

All variables are described as means ± SE. To evaluate the microvascular responses caused by metabolic stimulation or sodium nitroprusside, the percent change in diameter from baseline was calculated. Regression analysis (polynomial) was performed to assess the relationships between the microvascular diameters and the vessel sizes in each group. Statistical analysis of hemodynamics and microvascular diameter during each protocol was performed by analysis of variance for repeated measures. When significant values were obtained, Student’s t-test for paired samples (corrected for multiple comparisons with the Bonferroni inequality adjustment) was used to determine the measurements that significantly differed from one another (49). To compare the percent changes in microvascular diameters induced by metabolic stimulation between the groups, Student’s t-test for unpaired samples was applied. When the P value was <0.05, the differences were accepted as significant.

**Fig. 1.** The effect of metabolic stimulation by rapid pacing on myocardial oxygen consumption (MV˙O₂, A) and mean coronary flow velocity (B). There are no significant differences (NS) in the changes in MV˙O₂ and mean coronary flow velocity between vehicle- (Krebs solution) (open bars) and PTX-treated groups (solid bars).
and after the treatment with vehicle or PTX. Before the hearts were treated with vehicle or PTX, \( \dot{M}VO_2 \) was increased by 62 ± 8 and 66 ± 8%, respectively. After the hearts were treated, \( \dot{M}VO_2 \) was increased by 50 ± 5 and 48 ± 7%. The flow velocity was increased by 49.2 ± 5.9 and 44.9 ± 4.7% before the treatment with vehicle or PTX, respectively. After the hearts were treated, the mean flow velocity was increased by 38.7 ± 3.3 and 33.4 ± 2.9%. There were no significant differences in the changes of \( \dot{M}VO_2 \) and coronary flow velocity between the vehicle and PTX groups.

**Baseline Diameters at First and Second Measurements**

Table 2 shows the baseline diameters before and after vehicle and PTX treatments. The baseline diameters after vehicle and PTX treatments tended to decrease. However, baseline diameters of all size vessels before the second rapid pacing were not statistically different from those before the first rapid pacing in both groups.

**Dilation of Coronary Microvessels in Response to Metabolic Stimulation Before Treatment with Vehicle or PTX**

Figure 2 shows the diameter changes in response to metabolic stimulation before PTX or vehicle superfusion. The dilation of the coronary arterial microvessels was heterogeneous. Regression analysis revealed the size dependence of the metabolic stimulation-induced dilation. Metabolic stimulation caused greater vasodilation in the smaller coronary arterial microvessels in both groups.

**Dilation of Coronary Microvessels in Response to Metabolic Stimulation and to Sodium Nitroprusside After Treatment with Vehicle or PTX**

Figure 3 shows the diameter changes in response to metabolic stimulation after PTX or vehicle superfusion. As shown in Fig. 3A, the vasodilation in response to metabolic stimulation was clearly preserved in the vehicle group. In contrast, as shown in Fig. 3B, superfusion of PTX clearly abolished the dilation of coronary arterial microvessels in response to metabolic stimulation. Figure 3C shows the sodium nitroprusside-induced dilation after vehicle or PTX superfusion. There were no significant differences in sodium nitroprusside-induced dilation between the vehicle and PTX groups, suggesting that PTX does not attenuate the vasodilation reserve in a nonspecific manner.

**Comparison of Diameter Change during Rapid Pacing Between PTX and Vehicle Groups**

Figure 4 shows a comparison of the diameter changes between the PTX and vehicle groups during rapid pacing after each treatment. Vessels were divided into two groups according to the control diameters of the vessels: arterioles (internal diameter: \(<100 \mu m\)) and small arteries (internal diameter: \(>100 \mu m\)). Before arterioles were treated with vehicle or PTX, arterioles dilated by 8.6 ± 1.8% \((n = 10, 57 ± 8 \mu m \text{ at control})\) and 12.8 ± 2.5% \((n = 11, 66 ± 8 \mu m \text{ at control})\), respectively. After the arterioles were treated, as shown in Fig. 4A, the arterioles dilated by 16.1 ± 3.6% in response to rapid pacing in the vehicle group. However, arterioles in the PTX-treated group did not dilate \((-0.9 ± 1.6\%\). In the vehicle-treated group, there were no significant differences in the rapid pacing-induced dilation before and after vehicle treatment. Figure 4B shows the diameter changes of small arteries. In the vehicle-treated group, small arteries \((n = 8, 149 ± 18\%\)
μm at control) tended to dilate (2.9 ± 2.7%), but there were no significant differences from the control diameter. Small arteries in the PTX-treated group (n = 7, 166 ± 22 μm at control) did not dilate (−0.9 ± 2.0%).

Comparison of the Diameter Change during Rapid Pacing Between 8-Phenyltheophylline and Vehicle Groups

Table 3 shows the hemodynamics and blood gas data before and after the treatment with 8-phenyltheophylline. In this protocol, the blood pressure, blood gases, and blood pH did not change during 8-phenyltheophylline superfusion. Figure 5 compares the diameter changes during rapid pacing before and during the topical superfusion of 8-phenyltheophylline. There were no significant differences in the rapid pacing-induced dilation before (15.3 ± 2.0%) and during 8-phenyltheophylline treatment (16.3 ± 3.8%). Sodium nitroprusside dilated the vessels by 34.7 ± 6.0% after 8-phenyltheophylline treatment.

Figure 6 shows the effect of topically superfused 8-phenyltheophylline on the adenosine-induced arteriolar dilatation. Superfused adenosine produced a dose-dependent dilation of epicardial arterioles. Topically superfused 8-phenyltheophylline completely abolished the vasodilation by 10^{-8}–10^{-5} M of adenosine.

Effect of PTX on Cardiac Muscles

Figure 7 shows representative recordings of the force of cardiac muscles before and after the treatment with PTX. Forces were measured at 0.5 Hz stimulation and 5 min after 1 Hz stimulation. Table 4 shows the force and dF/dt of cardiac muscles before and after the treatment with PTX. There were no significant differences in force and dF/dt before and after PTX treatment for
1 h. There were also no significant differences in the percent increases in force and dF/dt in response to 1 Hz stimulation before and after PTX treatment, suggesting that PTX does not affect the contractility of myocardium.

**DISCUSSION**

The major finding of this study is that PTX abolished the dilation of coronary arterial microvessels in response to metabolic stimulation without altering the pacing-induced increase in MV˙O2, indicating that vasodilation during active hyperemia is critically mediated by GPTX. This study demonstrates for the first time that GPTX plays a crucial role in the active hyperemia of the coronary microcirculation.

**Critique of Methods**

PTX has been widely used to block Gi and Go proteins (6, 16, 45, 47). Our previous study (32) showed that

1. **Table 3.** Arterial blood gases, pH, and systemic hemodynamics in 8-PT treatment groups

2. **Fig. 5.** Microvascular response of each vessel caused by metabolic stimulation before and after 8-phenyltheophylline (8-PT) (10 μM) treatment (~) and the mean value before and after 8-phenyltheophylline treatment (~). Rapid pacing caused dilation of arterioles (15.3 ± 2.0% before and 16.4 ± 3.8% after treatment, n = 8 arterioles, 84.3 ± 11.0 μm at control). There were no significant differences in rapid pacing-induced dilations before and after 8-PT treatment. NS, not significant from before 8-PT.

3. **Fig. 6.** Adenosine dose-response curves with and without 8-PT. 8-PT (10 μM) significantly inhibited topically applied adenosine-induced vasodilation. n, no. of hearts.

4. The observed microvascular diameters did not change during the superfusion of PTX, for 2 h effectively blocks α2-receptor-mediated microvascular constriction, which is known to be exclusively mediated by Gα protein (32). This result indicated that superfusion of PTX for 2 h is sufficient to block GPTX of epicardial microvessels. It has been reported that intravenous injection of PTX effectively catalyzes the endogenous ADP-ribosylation of GPTX (Gαi and Gαo) proteins in the sarcolemma of excised hearts (15) and also inhibits the changes in vascular resistance caused by α2-agonists in pulmonary vascular beds (33). But neither of these studies evaluated the hemodynamic effect of PTX treatment. Our preliminary experiments with dogs showed that the intravenous treatment with PTX caused severe hemodynamic impairment, such as severe diastolic hypotension (unpublished data). Therefore, in the present study, we topically applied PTX to locally block GPTX in the epicardial coronary microvasculature and to avoid the involvement of the various other biological effects of PTX on the systemic circulation. Thus, in this study, the blood pressure, blood gases, and blood pH did not change during PTX superfusion. The observed microvascular diameters did not change during the superfusion of PTX, which is consistent with our previous studies (31, 32). We speculate that other PTX-insensitive mechanisms may have maintained the resting coronary microvascular tone. PTX treatment did not change the vascular responsiveness to sodium nitroprusside, suggesting that PTX does not attenuate the vasodilation reserve in a nonspecific manner. There were no significant differences in the percent increases in MV˙O2 between the vehicle and PTX groups. Therefore, the effect of PTX on the pacing-induced increase in myocardial metabolism seemed minimal. However, we did not measure changes in regional MV˙O2 but did measure changes in global MV˙O2. Therefore, it is possible that superfusion of PTX might locally reduce the developed tension and contractility of cardiac muscle, which,
Besides the heart rate, are the dominant determinants of MV\(\dot{O}_2\) of a beating heart. In such a case, MV\(\dot{O}_2\) will not increase during tachycardiac pacing. Therefore, we examined the direct effect of PTX on myocardium using trabeculae. We measured the developed force of cardiac muscle elicited by electrical stimulation. In the experiment, there were no significant differences in force and \(dF/dt\) during 0.5 Hz stimulation before and after PTX treatment. Also, there were no significant differences in the percent increases in force and \(dF/dt\) in response to 1 Hz stimulation before and after PTX treatment. These results suggest that the possibility of a direct effect by PTX on the myocardium is minimal.

In Fig. 1, the response in MV\(\dot{O}_2\) and, also, mean coronary flow velocity after vehicle and PTX treatment tended to decrease compared with measurements before treatment in both groups. It is possible that the pacing-induced response of the heart depended on the time course in our 2-h treatment of vehicle and PTX. However, there were no significant differences in the percent increases of MV\(\dot{O}_2\) (vehicle group: 62 ± 8% vs. 50 ± 5% and PTX group: 66 ± 8% vs. 48 ± 7%) and mean coronary flow velocity (vehicle group: 49 ± 6% vs. 39 ± 3% and PTX group: 45 ± 5% vs. 33 ± 3%) before and after vehicle and PTX treatment. In addition, in the vehicle group, the dilation of arterioles during the second rapid pacing tended to be greater than the dilation measured during the first rapid pacing, but the difference was statistically insignificant. Because we observed only epicardial arterioles, it may be possible that the response of epicardial arterioles slightly differed from the response of transmural coronary flow during rapid pacing.

**Activation of G\(_{PTX}\) During Metabolic Stimulation**

Receptor-dependent activation of G\(_{PTX}\). The trigger of G\(_{PTX}\) activation during rapid pacing-induced metabolic stimulation is not clear. G proteins play important roles in transducing various biological signals from the outside to the inside of the cell. Many endogenous substances that bind to their specific receptors on the surface of the cell membrane activate specific G proteins and modulate the activity of effectors such as ion channels and enzymes. These receptor-dependent signal transduction systems appear to be important pathways that mediate metabolic vasodilation. However, the agonist that mediates the microvascular responses via G\(_{PTX}\) during active hyperemia has not been determined. Adenosine might be a possible mediator. Adenosine has been known to activate G\(_i\) protein via A\(_1\) receptors in rat cardiomyocytes (28). Adenosine causes greater vasodilation in the smaller coronary arterial microvessels (24), similar to the metabolic stimulation observed in the present study. Furthermore, adenosine receptor agonists activated G\(_{PTX}\) via the A\(_2\) receptor in porcine coronary arterioles (21). However, the vasodepressor effect of adenosine, which is thought to be mediated by adenosine A\(_2\) receptors in peripheral resistance vessels, was not attenuated by PTX pretreatment in rats (18). In addition, the blockade of adenosine receptors failed to block the metabolic vasodilation in dogs (5, 35). Bache et al. (5) also showed that the blockade of adenosine receptors does not affect exercise-induced coronary vasodilation in hearts from conscious dogs in the absence of ischemia. In the present study, 8-phenyltheophylline neither changed the control vessel diameter nor failed to block the rapid pacing-induced dilation of coronary arterioles. In addition, superfused 8-phenyltheophylline completely abolished the vasodilation by \(10^{-8}-10^{-5}\) M of adenosine. This result is consistent with a previous report (see Ref. 29). The interstitial adenosine concentration has been reported to be \(10^{-6}\) M in ischemic myocardium (48) and 72 nM in canine myocardium during rapid pacing (50). Thus superfusion of 10 \(\mu\)M of 8-phenyltheophylline

Table 4. Force and dF/dt of cardiac muscle before and after PTX treatment

<table>
<thead>
<tr>
<th></th>
<th>Before Treatment</th>
<th>After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 Hz stimulation</td>
<td>1 Hz stimulation</td>
</tr>
<tr>
<td>Force, mN/mm(^2)</td>
<td>25.3 ± 0.7</td>
<td>29.5 ± 1.3</td>
</tr>
<tr>
<td>% increase in force</td>
<td>16.6 ± 2.3</td>
<td>0.121 ± 0.006</td>
</tr>
<tr>
<td>dF/dt, mN-mm(^{-2})ms(^{-1})</td>
<td>38.0 ± 3.1</td>
<td>39.8 ± 3.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 5\) cardiac muscles. Force and the change in force over time (dF/dt) were measured at 0.5 Hz and 5 min after 1 Hz stimulation at 28.0 ± 0.1°C.
sufficiently blocked the effect of adenosine receptors at physiological and pathophysiological concentrations of adenosine. Therefore, the contribution of adenosine to metabolic vasodilation via $G_{PTX}$ activation is assumed to be minimal in a physiological condition. However, when some mechanisms that open K$_{ATP}$ channels are inhibited by glibenclamide (glyburide), a compensatory increase in the interstitial adenosine concentration may occur that can result in adenosine receptor-mediated activation of the K$_{ATP}$ channels during exercise. Therefore, it may be possible that adenosine activates $G_{PTX}$ in coronary arteries in such a condition.

Receptor-independent activation of $G_{PTX}$. Some evidence has raised the possibility that $G$ proteins also transduce signals in a receptor-independent manner (31, 41). The possibility that shear stress activates $G_{PTX}$ in the endothelium, leading to K$^+$ channel activation followed by nitric oxide production and vasodilation, has been reported (41). In addition, shear stress rapidly activated $G$ proteins in human endothelial cells, and the greater part of the activation was inhibited by PTX (20). These studies raise the possibility that receptor-independent activation of $G_{PTX}$ in the endothelium may modulate the coronary blood flow when coronary blood flow increases in response to metabolic stimulation. Therefore, shear stress may have partly contributed to the metabolic vasodilation in the present study.

A recent in vitro study (22) has indicated that acidosis induces coronary arteriolar dilation through a $G_{PTX}$-signaling pathway. If rapid pacing causes acidosis in the myocardium, the acidosis might be related to the rapid pacing-induced vasodilation through a $G_{PTX}$-signaling pathway. However, the tissue pH during rapid pacing has not yet been reported. Although we did not measure the tissue pH, there were no significant differences in the pH of the coronary sinuses between those measured before and after the rapid pacing in this study. Therefore, it is not likely that acidosis induces vasodilation by rapid pacing.

Contribution of $G_{PTX}$ to Metabolic Vasodilation

$G_{PTX}$ is known to couple with various effectors, including adenyl cyclase, K$^+$ channels, phospholipase C, and phospholipase A$_2$, and it has been suggested that one $G_{PTX}$ can couple with more than one effector at the same time, resulting in the regulation of more than one effector function. Recent reports (13, 23, 26, 43) have suggested that K$_{ATP}$ channels and/or nitric oxide may be involved in metabolic coronary vasodilation.

$G_{PTX}$ and l-arginine-nitric oxide pathway in endothelium of microvessels. Several studies have shown that an inhibitor of nitric oxide synthase significantly reduces the metabolic coronary vasodilation induced by rapid pacing in guinea pigs (46), dogs (13, 23), and humans (43), whereas others have shown the opposite results in dogs (35) and humans (12). Furthermore, despite the fact that N$^\text{G}$-nitro-l-arginine methyl ester (l-NNAME) decreased the coronary blood flow at baseline and during metabolic stimulation, l-NNAME did not change the myocardial lactate extraction rate, i.e., it did not cause myocardial ischemia (35). These results may suggest that nitric oxide is not primarily involved in the mechanism that mediates metabolic coronary vasodilation. In the present study, metabolic stimulation by rapid pacing predominantly dilated arterioles $<100$ $\mu$m in diameter, whereas our (31) previous study showed that the L-arginine-nitric oxide pathway mediates the dilation of small coronary arteries $>130$ $\mu$m in diameter when $G_{PTX}$ is activated with mastoparan. In addition, shear stress activates $G_{PTX}$ in the endothelium (17), leading to K$^+$ channel activation followed by nitric oxide production and vasodilation (41). Therefore, it is possible that metabolic stimulation primarily dilates arterioles via mechanisms other than the L-arginine-nitric oxide pathway and then induces flow-dependent vasodilation in small arteries. Therefore, the coupling of $G_{PTX}$ in the endothelium and nitric oxide may contribute to metabolic vasodilation secondarily to an increase in shear stress in small arteries due to the downstream arteriolar dilation.

$G_{PTX}$ and K$_{ATP}$ channel. The role of K$_{ATP}$ channels in the coronary circulation and microcirculation is well established. K$_{ATP}$ channels importantly modulate coronary microvascular resistance in response to reductions in perfusion pressure (30) and also mediate the dilation of arterial microvessels both in brief ischemia and reactive hyperemia (25, 40).

In metabolic coronary vasodilation, many studies have focused on the role of K$_{ATP}$ channels, with a variety of methods, in dogs. In most cases, investigators have demonstrated that K$_{ATP}$ channels play an important role in metabolic coronary vasodilation. Duncker et al. (9) examined the role of K$_{ATP}$ channels in coronary vasodilation during exercise in chronically instrumented dogs, and they also examined the contribution of the K$_{ATP}$ channels to metabolic coronary vasodilation under normal and restricted coronary blood flow (10, 11). In these studies, glibenclamide decreased coronary flow during exercise under normal and restricted coronary blood flow. Glibenclamide also prevented the increases in coronary blood flow caused by $\beta$-adrenoceptor stimulation (39) and pacing tachycardia (27), whereas Aversano et al. (4) showed that the pacing-induced coronary flow increase was unaffected by glibenclamide. In another study, the role of K$_{ATP}$ in metabolic coronary vasodilation was examined by a direct observation method (13). In that study, Embrey et al. (13) showed that the arteriole dilation by the combination of $\beta$-adrenergic stimulation and rapid atrial pacing was unaffected by glibenclamide.

We (31) recently reported that direct activation of $G_{PTX}$ causes vasodilation via the L-arginine-nitric oxide pathway and via hyperpolarization by K$_{ATP}$ channel activation in the coronary microcirculation in vivo. However, the L-arginine-nitric oxide pathway mediates the dilation only in large microvessels ($>130$ $\mu$m), whereas K$_{ATP}$ channel activation plays a central role in the dilation of arterioles $<130$ $\mu$m in diameter when $G_{PTX}$ is directly activated. Therefore, metabolic stimu-
ulation by rapid pacing seems to selectively activate G_{PTX} linked to K_{ATP} channels in arterioles.

**Clinical Implications**

G_{PTX}-mediated endothelium-dependent vasodilation is impaired in diseased conditions such as atherosclerosis and hypercholesterolemia (14, 44), and a recent study (42) has also shown that oxidized low-density lipoprotein, which initiates atherosclerosis, inhibits G_{i} protein function in the aortic endothelial cell. In experimental diabetic animals, G_{i} protein is downregulated (19). Metabolic dilatation of resistance coronary arteries in response to rapid atrial pacing is impaired in patients with atherosclerosis (37) and diabetes mellitus (38). Thus these reports suggest that, in some disease conditions, an impairment of the G_{PTX}-mediated mechanism may cause an attenuation of metabolic vasodilation. These data are consistent with the present result that G_{PTX} plays a major role in regulating the coronary microvascular tone during metabolic stimulation.

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