Inhibition of endothelium-dependent hyperpolarization by endothelial prostanoids in guinea-pig coronary artery

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1 In smooth muscle of the circumflex coronary artery of guinea-pig, acetylcholine (ACh, 10^{-6} M) produced an endothelium-dependent hyperpolarization consisting of two components. An initial component that occurs in the presence of ACh and a slow component that developed after ACh had been withdrawn. Each component of the hyperpolarization was accompanied by an increase in membrane conductance.

2 Indomethacin (5×10^{-6} M) or diclofenac (10^{-6} M), both inhibitors of cyclooxygenase, abolished only the slow hyperpolarization. The initial hyperpolarization was not inhibited by diclofenac nor by nitroarginine, an inhibitor of nitric oxide synthase.

3 Both components of the ACh-induced hyperpolarization were abolished in the presence of atropine (10^{-6} M) or high-K solution ($[K^+]_0 = 29.4$ mM).

4 The interval between ACh-stimulation required to generate an initial hyperpolarization of reproducible amplitude was 20 min or greater, but it was reduced to less than 5 min after inhibiting cyclooxygenase activity. Conditioning stimulation of the artery with substance P (10^{-7} M) also caused a long duration (about 20 min) inhibition of the ACh-response.

5 The amplitude of the hyperpolarization generated by Y-26763, a K^+ -channel opener, was reproducible within 10 min after withdrawal of ACh.

6 Exogenously applied prostacyclin (PGI_2) hyperpolarized the membrane and reduced membrane resistance in concentrations over 2.8×10^{-9} M.

7 At concentrations below threshold for hyperpolarization and when no alteration of membrane resistance occurred, PGI_2 inhibited the initial component of the ACh-induced hyperpolarization.

8 It is concluded that endothelial prostanoids, possibly PGI_2 , have an inhibitory action on the release of endothelium-derived hyperpolarizing factor.

Keywords: PGI_2 ; EDHF; autoregulation; hyperpolarization; coronary artery; endothelium

Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; EDH, endothelium-dependent hyperpolarization; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; PGI_2 , prostaglandin I_2 ; s.d., standard deviation; SP, substance P

Introduction

Endothelial factors such as endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor (EDHF) or prostacyclin (PGI_2) are involved in many agonist-induced vasodilatations, and contribution of these factors in the dilatation differ between agonists and vascular beds (Furchgott, 1984; Vanhoutte *et al.*, 1986; Suzuki & Chen, 1990; Garland *et al.*, 1995). EDRF may be nitric oxide (NO) or a related NO-containing substance and this factor dilates vascular smooth muscle mainly by an increase in production of cyclic GMP through activation of guanylate cyclase (Ignarro *et al.*, 1987; Moncada *et al.*, 1991). This factor has been reported to activate Ca^{2+} -sensitive K^+ -channels of smooth muscle cells in the rabbit aorta (Bolotina *et al.*, 1994) or glibenclamide sensitive K^+ -channels in the rabbit mesenteric artery (Murphy & Brayden, 1995). The chemical nature of EDHF remains unclear; in some arteries, this factor may be a metabolite of arachidonic acid produced through epoxigenase pathways with activation of enzyme cytochrome P450 mono-oxygenase (Hecker *et al.*, 1994; Campbell *et al.*, 1996; Popp *et al.*, 1996; Chen & Cheung, 1996), but in others this is not the

case (Corriu *et al.*, 1996; Vanheel & Van de Voorde, 1997; Chataigneau *et al.*, 1998). EDHF hyperpolarizes the membrane by activating charybdotoxin-sensitive K^+ -channels (Hashitani & Suzuki, 1997) and induces vasodilatation by inhibiting the influx of Ca^{2+} through voltage-sensitive Ca^{2+} -channels (Nelson *et al.*, 1990) or the production of second messenger inositol trisphosphate (Itoh *et al.*, 1992). PGI_2 increases production of cyclic AMP to induce vasodilatation (Gryglewski *et al.*, 1991) and evokes membrane hyperpolarization sensitive to glibenclamide (Parkington *et al.*, 1995).

In the main descending coronary artery of guinea-pig, ACh hyperpolarizes the membrane in an endothelium-dependent manner with three components, i.e., EDRF-, EDHF- and PGI_2 -induced components, and the former and the latter components are inhibited by nitroarginine and indomethacin respectively (Parkington *et al.*, 1993). It remains unclear how these endothelial products interact to each other. In circumflex coronary artery of the guinea-pig, we found that PGI_2 has inhibitory effects on the release of EDHF, and at higher concentrations hyperpolarizes the smooth muscle membrane. Some of the data was reported briefly in the International Society for Autonomic Neuroscience at Cairns, Australia (Yajima *et al.*, 1997).

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Methods

Guinea-pigs of either sex, weighing 200–250 g, were killed by decapitation after asphyxiation with CO₂ gas, and the heart removed. Proximal segments (about 5 mm) of the circumflex coronary artery to the left atrium (outer diameter, 0.1–0.3 mm) were isolated from the heart which was immersed in the oxygenated Krebs solution. The artery was cleaned by removing the surrounding cardiac and connective tissues and mounted in an experimental chamber. The chamber was 8 mm wide, 8 mm deep and 3 cm long, with a rubber plate at the bottom. The artery was pinned down on the rubber plate whilst it was superfused for over 1 h before starting experiments with Krebs solution warmed to 35°C, at a constant flow rate of about 3 ml min⁻¹, so as to maintain the volume of the solution in the chamber at about 0.5 ml. In some vessels, the endothelial cells were damaged by the internal perfusion with distilled water for 15–20 min.

Artery was stimulated with ACh, usually for 1 min, by including ACh in the superfusing solution. Inhibitors of NO-synthase or cyclooxygenase were added to the superfusate 10 min before stimulation with ACh, during which time the membrane potential of the smooth muscle cells was recorded.

Conventional intracellular microelectrode techniques were used to measure the membrane potentials of smooth muscle cells in the coronary arteries. Briefly, a glass capillary microelectrode filled with 3 M KCl (tip resistance; 50–70 MΩ) was inserted into smooth muscle cells from the adventitial side of the vessel. Criteria for successful penetration of the electrode into a smooth muscle cell were a sharp deflection of potential to about -50 mV and the maintenance of a stable membrane potential of between -45 to -55 mV for over 3 min.

The end of an isolated vessel was drawn into a glass capillary with tip diameter of 100–200 μm and was stimulated by rectangular current pulses of 2–3 s duration and up to 0.64 mA intensity through the capillary. Electrotonic potentials thus produced by extracellular stimulation were recorded in close proximity to the edge of the capillary (usually less than 0.1 mm).

The ionic composition of the Krebs solution was as follows (with mM); Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134 and glucose 11.5. Solution containing 29.4 mM [K⁺]₀ (high-K solution) was prepared by replacing NaCl with KCl. The solution was oxygenated (95% O₂, 5% CO₂) and had a pH of 7.2–7.3.

Drugs used were acetylcholine chloride (ACh), atropine sulphate, indomethacin and diclofenac sodium salt (all from Sigma, U.S.A.), substance P and N^ω-nitro-L-arginine (nitroarginine, Peptide Institute, Osaka, Japan), Y-26763 (Yoshitomi Pharmac., Osaka, Japan) and prostacyclin (PGI₂, Ono Pharmaceutical Ind., Ltd., Osaka, Japan). These drugs were dissolved in distilled water (ACh, atropine, diclofenac, substance P, nitroarginine), 95.5% ethanol (PGI₂), dimethyl sulphoxide (Y-26763) or 5 × 10⁻³ M Na₂CO₃ solution (indomethacin), and diluted further with Krebs solution to obtain desired concentrations. The final concentration of the solvent did not exceed 1:1000 in the Krebs solution. Addition of drugs or their vehicle to the Krebs solution did not alter the pH of the solution.

The measured values were expressed as the mean ± s.d.mean. Statistical significance was tested using Student's *t*-test, and probabilities of less than 5% (*P* < 0.05) were considered significant.

Results

Endothelium-dependent hyperpolarization produced by ACh

In smooth muscle cells of the circumflex coronary artery of the guinea-pig, the resting membrane potential was stable, ranging between -45 and -55 mV (-52.2 ± 3.5 mV, *n* = 52 arteries). Stimulation of the artery with acetylcholine (ACh, 10⁻⁶ M) for 1 min produced a hyperpolarization with two components. The hyperpolarization appeared during application of ACh (an initial hyperpolarization) and reached a peak amplitude ranging between 15 and 25 mV (19.5 ± 5.5 mV, *n* = 35 arteries) within 15–20 s. The amplitude of the initial hyperpolarization remained constant or often declined gradually by 10–30% during application of ACh. The initial hyperpolarization was followed by a slow hyperpolarization which appeared during the recovery process of the initial hyperpolarization and reached a peak amplitude of 1–8 mV (4.3 ± 1.2 mV, *n* = 35 arteries), 2–4 min after withdrawal of ACh. The duration of the slow hyperpolarization measured after withdrawal of ACh ranged between 5 and 8 min (Figure 1A). Both components of the ACh-induced hyperpolarization were absent after inhibiting muscarinic receptors with 10⁻⁶ M atropine (*n* = 5 arteries, data not shown).

The threshold concentrations of ACh required for generation of the initial and slow components of the hyperpolarization were 10⁻⁸ M and 10⁻⁷ M, respectively. The amplitude of each component of the hyperpolarization increased in a concentration-dependent manner to reach a maximum value of about 20 and 4 mV for the initial and slow hyperpolarizations, respectively, at 10⁻⁶ M (Nishiyama *et al.*, 1998). These properties of the hyperpolarization were essentially the same as those reported by Parkinson *et al.* (1993) in the left descending main coronary artery.

In intact arteries, application of diclofenac (10⁻⁶ M) for over 5 min abolished the slow component of the ACh-induced hyperpolarization, with no significant alteration of the membrane potential (-51.2 ± 2.8 mV, *n* = 15 arteries, *P* > 0.1) or the amplitude of the initial hyperpolarization (20.2 ± 3.8 mV, *n* = 10 arteries; *P* > 0.1) (Figure 1A and B). Indomethacin (5 × 10⁻⁶ M) had effects similar to diclofenac; that is, having no significant effect on the membrane potential (-51.8 ± 2.5 mV, *n* = 8 arteries, *P* > 0.1) or the amplitude of the initial hyperpolarization (19.6 ± 4.8 mV, *n* = 8 arteries, *P* > 0.1) and inhibiting only the slow component in the intact coronary artery (*n* = 8 arteries, see Figure 3). Nitroarginine (10⁻⁵ M) added in the presence of diclofenac did not further alter the membrane potential (-51.8 ± 2.1 mV, *n* = 12 arteries, *P* > 0.1) or the initial component of the hyperpolarization (20.1 ± 4.5 mV, *n* = 6 arteries; *P* > 0.1) (data not shown).

The lumen of the arteries were perfused with distilled water for 20–30 min to damage endothelial cells (four arteries). This procedure caused membrane depolarization by 10–15 mV (-40.5 ± 2.0 mV, *n* = 12 cells, *P* < 0.05), and the change in membrane potential produced by 10⁻⁶ M ACh was negligible (1–3 mV hyperpolarization, *n* = 3 arteries; 2.5 mV depolarization, *n* = 1 artery), suggesting that the endothelial cells were successfully damaged. The results indicate that the ACh-induced hyperpolarization was generated in an endothelium-dependent manner.

In two circumflex coronary arteries with endothelium intact, high-K solution ([K⁺]₀ = 29.4 mM) depolarized the membrane by about 15 mV (from -50.2 ± 1.5 mV, *n* = 5, to -34.4 ± 2.4 mV, *n* = 5), reduced the amplitude of initial component of the ACh-induced hyperpolarization to

Figure 1 Inhibition by diclofenac of the slow component of the acetylcholine (ACh)-induced hyperpolarization. The circumflex coronary artery of the guinea-pig was stimulated with ACh (10^{-6} M) for 1 min before (A, control) and after application of 10^{-6} M diclofenac (B), or during depolarization with high-K solution ($[K^+]_0 = 29.4$ mM) (C). The dotted lines in A and B represent the level of the resting membrane potential. A, B and C were recorded from different tissues.

Figure 2 Reduction of electrotonic potentials during the acetylcholine (ACh)-induced hyperpolarization. A coronary artery was stimulated with five sets of rectangular depolarizing and hyperpolarizing current pulses of increasing intensities (2 s duration) at 10 s interval. Electrotonic potentials produced by these stimuli were recorded from the smooth muscle located 0.1 mm from the edge of the capillary before (A), during application of 10^{-6} M ACh (B), at 3 min (C) and 12 min (D) after removal of ACh. Intensity of stimuli for A–D: 0.1, 0.2, 0.31, 0.41 and 0.58 mA. (E) Current-voltage relationships recorded before (Control, ○), during application of 10^{-6} M ACh (△) and after washing ACh for 3 min (▲) and 13 min (●) from the smooth muscle. Amplitudes of electrotonic potentials were plotted as a function of the intensity of stimuli (positive value, outward current; negative value, inward current). All points were recorded from the same cell. A–D are continuous recordings. A–D and E were recorded from different tissues.

1.1 ± 0.1 mV ($n=5$) and abolished the slow component (Figure 1C).

Ionic conductance of the membrane during the ACh-induced hyperpolarization

Extracellular electrical stimulation of the coronary artery with rectangular current pulses (2 s duration, 0.06–0.64 mA intensity) evoked electrotonic potentials in smooth muscle cells located in close proximity (0.1–0.3 mm) to the edge of the suction electrode. In three arteries, the amplitude of the electrotonic potentials produced by alternate application of

inward and outward current pulses at constant intensity (0.1–0.3 mA) and frequency (0.1 Hz) was found to be reduced during the ACh-induced hyperpolarization, indicating that the ACh-induced hyperpolarization was produced by an increase in ionic conductance of the membrane.

The relative changes in membrane resistance during the ACh-induced hyperpolarization were calculated by applying the cable equation (Hodgkin & Rushton, 1946). Provided internal resistances including gap junctional resistance remain the same before and during the ACh-induced hyperpolarization and distance between stimulating and recording electrodes (less than 0.1 mm) is much smaller than the length constant of

Figure 3 Diminished hyperpolarization produced by repeated application of acetylcholine (ACh) and its recovery by indomethacin. ACh (10^{-6} M) was applied for 1 min at 10 min interval, in the absence (A) and presence of 5×10^{-6} M indomethacin (B). (C) time scale in min with 0 starting at the time of withdrawal of the first ACh application. Dotted line in A, the resting potential level of -50 mV. Each pair of responses shown in A and B was recorded from single cells in different tissues. The lower graph (D) shows the relationship between the amplitude of hyperpolarization produced by the second ACh application (relative to the first ACh-response) and the interval between two ACh-stimuli, measured in the absence (●) and presence (○) of 5×10^{-6} M indomethacin. Data obtained from five tissues were plotted.

the tissue (assuming about 1.0 mm), then the ratio of the amplitude of electrotonic potential $(V'/V)^2 \doteq \gamma_m'/\gamma_m$ (Suzuki & Kuriyama, 1975), where V and V' or r_m and r_m' are the amplitude of the electrotonic potentials produced by a given intensity of current or the membrane resistances at rest and during the ACh-induced hyperpolarization, respectively. The changes in membrane resistance thus calculated were 0.29 ± 0.11 ($n=4$) for the initial component and 0.71 ± 0.20 ($n=4$) for the slow component of the ACh-induced hyperpolarization.

The ionic mechanisms involved were further investigated from the current-voltage relationships produced by current pulses of different intensities recorded in the absence and presence of ACh. Before application of ACh, the amplitude of electrotonic potentials produced by inward current pulses increased linearly with an increase in stimulus intensity. Amplitude of electrotonic potentials produced by outward current pulses also increased linearly with intensity of stimuli until the membrane potential reached about -40 mV, and the relationship became dissociated from the linear line at stronger intensities (i.e., rectification). Outward current pulses at higher intensities (usually >0.3 mA) evoked a spike potential of amplitude $5-15$ mV at the on-set of the electrotonic potential (Figure 2A). The amplitudes of electrotonic potentials were smaller in the presence than in the absence of ACh (Figure 2A and B). This was also the case when electrotonic potentials were produced 3 min after withdrawal of ACh, i.e., the amplitude of electrotonic potential was reduced during the slow hyperpolarization but less so than during the initial hyperpolarization (Figure 2C). The electrotonic potentials had returned to control amplitudes 12 min after withdrawal of ACh when the membrane potential returned to the control level (Figure 2D).

The peak amplitudes of electrotonic potential obtained in different conditions were plotted on the same scale as a function of the intensity of stimuli (Figure 2E), and the relationship was found to be linear in the absence or presence of ACh, but the slope was reduced during the ACh-induced fast and slow hyperpolarizations. The plot obtained in the presence of ACh crossed that obtained before application of ACh at -81.8 ± 15.6 mV ($n=4$ arteries), while the plot obtained at 3 min after withdrawal of ACh (i.e., during the slow hyperpolarization) crossed at -85.0 ± 8.8 mV ($n=6$ arteries). These two values were not significantly different ($P>0.1$), indicating that both hyperpolarizations had reversed potentials similar to the equilibrium potential for K^+ in arterial smooth muscles (approximately -83 mV, Casteels *et al.*, 1977). Thus, the initial and slow components of the ACh-induced hyperpolarization are produced mainly by an increase in conductance of the membrane to K^+ . The slope of the current-voltage relationship measured 12 min after withdrawal of ACh from the superfusate had returned to the control level, indicating the complete recovery of the resting ionic conductance of the membrane.

Reproducibility of the hyperpolarization produced by repeat stimulation with ACh

In the absence of any inhibitory drugs, a repeat stimulation of the artery with ACh at intervals shorter than 20 min caused a reduction in amplitude of the second response. In the cell shown in Figure 3A, the duration of slow hyperpolarization produced by the first application of ACh was about 6.5 min. The amplitude of the initial and slow hyperpolarizations produced by a second application of ACh 10 min later was $75 \pm 9\%$ and $70 \pm 16\%$ ($n=3$) of the first, respectively.

Experiments were carried out to determine the time required to produce ACh-induced hyperpolarization of reproducible amplitude. It was found that a reproducible amplitude of the initial component of the hyperpolarization was generated when the interval of ACh-stimulation was about 20 min (Figure 3D). The recovery of the slow component of the hyperpolarization was variable, requiring 10–35 min, and therefore the studies on the slow component were not further carried out. Similar experiments were made during application of 5×10^{-6} M indomethacin to inhibit the generation of slow hyperpolarization. It was found that the amplitude of hyperpolarization produced by the second application of ACh was now no longer significantly smaller than the first (Figure 3B; $98 \pm 8\%$ of the initial hyperpolarization, $n=3$) when it was applied at intervals of 5 min or longer (Figure 3D).

These results suggest that the slow hyperpolarization is produced by endothelial prostanoids synthesized through activation of cyclooxygenase, and that the prostanoids have inhibitory actions on the generation of the initial component of the ACh-induced hyperpolarization. Attempts were made to observe whether the prostanoids produced by agonists other than ACh were also effective in inhibiting the generation of the ACh-induced hyperpolarization. In circumflex coronary artery of the guinea-pig, substance P (SP, 10^{-7} M) also hyperpolarized the membrane with two components; in 15 cells from five arteries, the initial hyperpolarization reached the peak amplitude of 20.5 ± 3.5 mV from the resting potential after which it gradually decayed in amplitude, while the second slow hyperpolarization reached the peak amplitude of 5.3 ± 1.2 mV at 3–4 min after withdrawal of SP, and it required 5–8 min (6.8 ± 0.5 min) for complete recovery (Figure 4B). Application of indomethacin (5×10^{-6} M) or diclofenac (10^{-6} M) inhibited only the slow hyperpolarization (data not shown), indicating that this component had properties similar to the slow hyperpolarization produced by ACh.

ACh (10^{-6} M) was applied two times, at least 20 min before and various times after application of 10^{-7} M SP, and the amplitudes of hyperpolarization produced by these two ACh applications were compared. When the second ACh was applied 6 min after withdrawal of SP, the amplitude of the initial and slow components of the hyperpolarization was about 60 and 70% of the first, respectively (Figure 4A and B). The group data (Figure 4C) indicated that a reproducible amplitude of the initial component of the hyperpolarization could be elicited when ACh was applied longer than about 20 min after withdrawal of SP. The effects of conditioning stimulation with SP on the slow component of the ACh-induced hyperpolarization were not investigated, due to unsuccessful measurement of this potential consistently. Thus, these results indicate that the endothelial prostanoids have inhibitory actions on the release of EDHF, irrespective of the agonists used to release the prostanoids.

Hyperpolarization produced by Y-26763

Experiments were carried out to observe the effects of conditioning stimulation of the coronary artery with ACh on the hyperpolarization responses produced by Y-26763, a K^+ -channel opener (Itoh *et al.*, 1994), the objective being to determine possible effects of endothelial prostanoids on the hyperpolarizing responses of arterial smooth muscle. In six arteries in the absence of any stimulation, application of 10^{-6} M Y-26763 for 2 min hyperpolarized the membrane for about 20 min, with a peak amplitude of 12.5 ± 1.5 mV, and a similar hyperpolarization with a peak amplitude of 11.5 ± 2.5 mV ($P>0.1$) was also elicited when this concentra-

Figure 4 Inhibition of the acetylcholine (ACh)-induced hyperpolarization by conditioning stimulation with substance P. ACh (10^{-6} M) was applied for a duration of 1 min, 25 min before (A) and 6 min after 1 min stimulation of tissue with 10^{-7} M substance P (SP) (B). A and B were recorded from the same cell. (C). The relationship between amplitude of the initial hyperpolarization produced by the second ACh application (relative to the first) and the interval between withdrawal of 10^{-7} M substance P and the second application of ACh. Data obtained from three tissues were plotted.

tion of Y-26763 was applied 10 min after stimulation with ACh. The results indicate that the reduction of the ACh-induced hyperpolarization for periods 10 min or greater is not due to a failure of the smooth muscle membrane of the coronary artery to produce hyperpolarization.

Effects of PGI₂ on smooth muscle of the coronary artery

In smooth muscle of the guinea-pig coronary artery, application of PGI₂ ($>2.8 \times 10^{-9}$ M) hyperpolarized the membrane. A solution containing an ethanol concentration equivalent to that used to make up a solution of 2.8×10^{-6} M PGI₂ did not produce any detectable change in the membrane potential ($n=3$ arteries, data not shown). The PGI₂-induced hyperpolarization remained unaltered in the presence of 5×10^{-6} M indomethacin, 10^{-6} M diclofenac or 10^{-5} M nitroarginine ($n=3$ arteries, data not shown), indicating that the generation of the response did not involve activation of cyclooxygenase or NO synthase and reflects a direct action on smooth muscle. Figure 5A shows the relationship between concentration of PGI₂ and amplitude of hyperpolarization in smooth muscle of the guinea-pig coronary artery. PGI₂ (2.8×10^{-9} – 2.8×10^{-6} M) hyperpolarized the membrane in a

concentration-dependent manner, and the maximum value was obtained at 8.4×10^{-7} M. The amplitude of electrotonic potentials produced by alternate stimulation of the artery with inward and outward current pulses of constant intensity was reduced during the PGI₂-induced hyperpolarization (Figure 5B), indicating that the potential was produced by an increase in ionic conductance of the membrane. Changes in the membrane resistance during the PGI₂-induced hyperpolarization were calculated, according to the cable theory (Hodgkin & Rushton, 1946), and the reduction was 0.61 ± 0.24 ($n=5$) times the control.

Electrotonic potentials were evoked by current steps of increasing intensity (0.1–0.6 mA) in the absence and presence of 8.4×10^{-8} M PGI₂, and the relationship was plotted on the same scale. The relationship was linear for both control and test cases, with the slope less steep in the presence than in the absence of PGI₂ (Figure 5C). The current-voltage relationships crossed at -85.0 ± 8.5 mV ($n=3$ arteries), suggesting that the PGI₂-induced hyperpolarization was produced mainly by an increase in conductance of the membrane to K⁺.

Electrical responses produced by PGI₂ were compared with those of the ACh-induced hyperpolarization in smooth muscle

Figure 5 PGI₂-induced hyperpolarization accompanied by a reduction in amplitude of electrotonic potentials. (A) The relationship between concentration of PGI₂ and amplitude of hyperpolarization recorded in the arterial smooth muscle is shown by the means $\pm$ s.d. mean ($n=4-12$). Peak amplitude of the hyperpolarization produced by application of PGI₂ for 3 min was measured. Data obtained from seven tissues are summarized. Significant hyperpolarization was observed at $>2.8 \times 10^{-9}$ M PGI₂. (B) A circumflex coronary artery was stimulated with 2.8×10^{-8} M PGI₂ for 1 min (shown by a bar above the record) while inward and outward current stimuli of a constant intensity (0.26 mA) were applied alternatively to produce electrotonic potentials. (C) Current-voltage relationships recorded before (Control, $\circ$) and during application of 8.4×10^{-8} M PGI₂ ($\bullet$). Amplitudes of electrotonic potentials were plotted as a function of the intensity of stimuli (positive value, outward current; negative value, inward current). All points were recorded from the same cell.

of the guinea-pig. In the hyperpolarizations produced by application of 10^{-6} M ACh for 1 min ($n=4$ cells), the initial component had a latency of onset of 18.1 ± 3.3 s and peak amplitude of 25.5 ± 5.0 mV while the slow hyperpolarization had a peak amplitude of 5.1 ± 2.2 mV and duration of 6.0 ± 0.9 min after withdrawal of ACh (Figure 6A). In the presence of 10^{-6} M diclofenac ($n=3$ cells), ACh produced only an initial hyperpolarization with a latency of 17.8 ± 5.1 s and amplitude of 24.8 ± 3.8 mV (Figure 6B). Application of 8.4×10^{-8} M PGI₂ for 1 min also hyperpolarized the membrane by 6.5 ± 3.1 mV ($n=5$ cells), the value being similar to that produced by ACh, but with a slow time course taking 25–40 s (30.3 ± 8.5 s) to reach the peak amplitude and lasting 4–8 min (5.5 ± 0.7 min) (Figure 6C). The duration of the PGI₂-induced hyperpolarization was not significantly different from the ACh-induced slow hyperpolarization.

ACh (10^{-6} M) and PGI₂ (8.4×10^{-8} M) when applied simultaneously after blockade of cyclooxygenase by diclofenac induced a response which was similar to that produced by ACh in the absence of diclofenac (Figure 6A and B), although the amplitude of the initial hyperpolarization was reduced by about 20% (20.2 ± 2.8 mV, $n=3$ cells). Thus, the slow hyperpolarization produced by ACh could be mimicked by exogenously applied PGI₂.

Possible inhibitory actions of exogenously applied PGI₂ on the ACh-induced hyperpolarization were examined. As shown in Figure 7, application of 2.8×10^{-10} M PGI₂, which itself was without effect on the membrane potential, reduced the amplitude of ACh-induced hyperpolarization to about 60%. In five experiments, the amplitude of the ACh-induced hyperpolarization was significantly reduced to $89.1 \pm 8.8\%$ by 2.8×10^{-11} M PGI₂ and to $77.6 \pm 8.7\%$ by 2.8×10^{-10} M PGI₂. In three experiments, 2.8×10^{-9} M PGI₂ hyperpolarized the membrane by 1.8 ± 0.5 mV and reduced the ACh-induced hyperpolarization to $50.2 \pm 6.1\%$. The inhibitory actions of PGI₂ on the ACh-induced hyperpolarization were also observed in the presence of diclofenac; 2.8×10^{-11} M PGI₂ reduced the initial component of the ACh-induced hyperpolarization to $88.1 \pm 6.9\%$ ($n=3$ cells, $P<0.05$, Figure 7C and D). Thus, PGI₂ in concentrations below threshold for hyperpolarization of smooth muscle membrane inhibits the ACh-induced hyperpolarization.

Discussion

The present experiments have shown that in the circumflex coronary artery of guinea-pig, ACh produces a hyperpolariza-

Figure 6 Simulation of the acetylcholine (ACh)-induced slow hyperpolarization by exogenously applied PGI_2 . In the guinea-pig coronary artery, membrane responses produced by ACh and PGI_2 were recorded in the absence (A) and presence of 10^{-6} M diclofenac (B–D). ACh 10^{-6} M (A, B and D) or 8.4×10^{-8} M PGI_2 (C) was applied for 1 min at intervals 20–25 min. A–D were recorded from the same cell.

tion with two components, in an endothelium-dependent manner. The ACh-induced response was abolished after application of atropine, suggesting that the response was produced as a result of stimulation of muscarinic receptors by ACh. Parkinson *et al.* (1993) reported that in the left descending main coronary artery of guinea-pig the ACh-induced response is separated into three components, as the initial hyperpolarization produced by EDHF and the slow hyperpolarization consists of EDRF- and prostanoids-induced components. We found that the slow hyperpolarization was produced by endothelial prostanoids, as this component disappeared completely after inhibiting the activity of cyclooxygenase with indomethacin or diclofenac but not by nitroarginine. The initial component was not sensitive to nitroarginine, indomethacin or diclofenac. Thus, only two components, a nitroarginine and indomethacin-resistant component (possibly produced by EDHF) and indomethacin-sensitive component (produced by prostanoids) were confirmed in all vessels used in the present experiments.

The main finding of the present experiments is the inhibitory actions of endothelial prostanoids on the initial

component of the ACh-induced hyperpolarization. While the duration of the slow hyperpolarization is 5–7 min, the recovery from the inhibition of the ACh-induced hyperpolarization requires over 20 min. Thus, the membrane potential and membrane resistance revert to the resting level before cessation of the inhibitory action on the EDHF-induced hyperpolarization, indicating that the delayed recovery of the ACh-induced response is not due to the changes in biophysical properties of smooth muscle membrane. We speculate that the endogenously released prostanoids inhibit the release of EDHF by autocrine or paracrine actions. The significant shortening of the recovery time for the ACh-response by exposure to a cyclooxygenase inhibitor strongly supports this idea. Furthermore, the inhibitory actions of endogenous prostanoids on the ACh-response could be mimicked by subthreshold concentrations of exogenously applied PGI_2 (equal to $<2.8 \times 10^{-9}$ M). An important function of endothelial PGI_2 is the prevention of blood coagulation by inhibition of the adhesion of platelets to the vessel wall (Gryglewski *et al.*, 1991). We would like to propose that the inhibitory actions on the release of EDHF should also be counted into the role of

Figure 7 Inhibition of the acetylcholine (ACh)-induced hyperpolarization by subthreshold concentrations of PGI₂. ACh (10^{-6} M) was applied for 1 min at intervals 20–25 min, in the absence (A or C) and presence of 2.8×10^{-10} M PGI₂ (B) or 2.8×10^{-11} M PGI₂ (D). Responses were recorded in the absence (A and B) and presence of 10^{-6} M diclofenac (C and D). A–B and C–D were recorded from single cells in the same tissue.

endothelial PGI₂. This prostanoid is a potent vasodilator in some vessels (Parkington *et al.*, 1995; Zhang *et al.*, 1996), and therefore the inhibitory actions of PGI₂ on the release of EDHF would weaken the endothelium-dependent vasodilatation. The physiological meaning of the inhibitory actions of endothelial PGI₂ on the release of EDHF remains unclear.

Endothelial cells are large source of PGI₂ during stimulation with agonists including ACh (Gryglewski *et al.*, 1991), and in some arteries this prostanoid hyperpolarizes smooth muscle membrane by activating glibenclamide-sensitive K⁺-channels (Siegel *et al.*, 1992; Parkington *et al.*, 1995; Yajima *et al.*, 1997). The present experiments confirmed that the hyperpolarization is indeed accompanied by an increase in K⁺ conductance. In cardiovascular tissues, glibenclamide is reported to inhibit the ATP-sensitive K⁺-channels (Nelson & Quayle, 1995). However, glibenclamide may have additional actions to inhibit prostanoid receptors (Zhang *et al.*, 1991), indicating that contribution of the ATP-sensitive K⁺ channels in the PGI₂-induced hyperpolarization cannot be determined from the effects of glibenclamide alone. The initial hyperpolarization was, on the other hand, inhibited by charybdotoxin (Yajima *et al.*, 1997; Nishiyama *et al.*, 1998), indicating that these two components of the hyperpolarization were generated by activation of different types of K⁺-channels.

The present experiments showed that the time course of the hyperpolarization produced by exogenously applied PGI₂ is similar to that produced by endothelial prostanoids. This property of PGI₂ allowed us to mimic the ACh-induced slow hyperpolarization by exogenous application of PGI₂. These observations suggest that the slow onset and time course of the hyperpolarization produced by endothelial prostanoids are not due to the delayed onset and sustained activation of prostanoid-synthesis systems after withdrawal of ACh, but

probably due to the properties of the prostanoid response. Although the present experiments could not determine the chemical nature of the endothelial prostanoids, the observations strongly suggest that the slow hyperpolarization is produced by PGI₂. This is also supported by the evidence that both the slow hyperpolarization and PGI₂-induced hyperpolarization are inhibited by glibenclamide (Parkington *et al.*, 1995; Yajima *et al.*, 1997). On this assumption, the concentration-response relationship of the PGI₂-induced hyperpolarization allows estimation that the amount of PGI₂ released from endothelial cells in response to 10^{-6} M ACh is in the range of 2.8×10^{-9} – 2.8×10^{-8} M.

The endothelium-dependent hyperpolarization (EDH) is produced by a humoral substance, EDHF, in the coronary artery of guinea-pig (Chen *et al.*, 1991). In the porcine coronary artery, EDHF may be eicosatrienoic acids metabolized from arachidonic acid (Hecker *et al.*, 1994; Popp *et al.*, 1996). However, electrical coupling between endothelial and smooth muscle cells through gap junctions has also been demonstrated in the rat aorta (Marchenko & Sage, 1996), porcine coronary artery (von der Weid & Beny, 1993) and submucosal arterioles of the guinea-pig (Yamamoto *et al.*, 1998). These findings suggest that EDH is produced by an electrotonic spread of potentials from the endothelial cells. However, the rectification of signal transmission noted in the gap junctions between endothelial and smooth muscle cells of the porcine coronary artery, possibly because of electrical mismatch with the large smooth muscle syncytium and the smaller endothelial syncytium (Beny & Pacicca, 1994), suggest that the properties of EDH differ between large and small vessels.

In conclusion, endothelial prostanoids (possibly PGI₂) released by agonists hyperpolarize the smooth muscle membrane by activating K⁺-channels and also inhibit the release of EDHF. Endothelial prostanoids may have inhibitory

actions on the myoendothelial gap junctions, if EDH is produced by the electrotonic spread of potentials from the endothelial cells. The inhibitory actions of EDH appears below threshold concentration to hyperpolarize smooth muscle membrane.

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