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Inhibitors of the cytochrome P450-mono-oxygenase and endothelium-dependent hyperpolarizations in the guinea-pig isolated carotid artery

Catherine Corriu, Michel Félétou, Emmanuel Canet & [1,]*Paul M Vanhoutte

Département de Pneumologie, Institut de Recherches Servier 11, rue des Moulineaux 92150 Suresnes and *Institut de Recherches Internationales Servier, 6 place des Pléiades, 92410 Courbevoie, France

1 Transmembrane potentials were recorded from isolated carotid arteries of the guinea-pig superfused with modified Krebs-Ringer bicarbonate solution. Smooth muscle cells were impaled with sharp intracellular microelectrodes.

2 Acetylcholine (1μM) induced an endothelium-dependent hyperpolarization (14.3±2.8 mV, n=6) which was not affected (15.1±1.1 mV, n=35) by inhibitors of cyclo-oxygenase (indomethacin, 5 μM) and nitric oxide synthase (N^{ω}nitro-L-arginine:L-NOARG, 100 μM).

3 The hyperpolarization produced by acetylcholine was abolished in the presence of elevated potassium (35 mM) in the superfusing physiological saline solution.

4 The acetylcholine-induced hyperpolarization was not affected by the inhibitors of cytochrome P450 mono-oxygenases, SKF525a (10 and 100 μM, 13.9±2.2 and 15.3±4.6 mV), metyrapone (100 μM, 13.1±1.9 mV), clotrimazole (100 μM, 13.5±2.7 mV), 17-octadecynoic acid (5 μM, 16.5±1.9 mV), methoxsalen (10 μM, 15.3±1.6 mV), the inhibitor of phospholipase A_2 quinacrine (10 μM 12.8±2.5 mV) and the non specific lipoxygenases/cyclo-oxygenases/cytochrome P450 inhibitor, eicosatetraynoic acid (50 μM, 15.0±2.2 mV). However, the muscarinic antagonist, atropine (100 nM), abolished the hyperpolarization.

5 These results suggest that in guinea-pig carotid artery, the metabolism of arachidonic acid, either through cyclo-oxygenase, lipoxygenase or cytochrome p450 mono-oxygenase, is not involved in acetylcholine-induced endothelium-dependent hyperpolarizations.

Keywords: EDHF; endothelium; hyperpolarization; electrophysiology; cytochrome P450; clotrimazole; SKF525a; metyrapone; quinacrine; smooth muscle cells

Introduction

Acetylcholine relaxes vascular smooth muscle by releasing various diffusible substances (see review Furchgott & Vanhoutte, 1989). Nitric oxide (Palmer *et al.*, 1987) produced from L-arginine by nitric oxide synthase evokes vascular smooth muscle relaxation by activation of soluble guanylate cyclase (Rappoport & Murad, 1983). Another substance, endothelium-derived hyperpolarizing factor (EDHF), hyperpolarizes the smooth muscle cells by stimulating potassium conductance (Félétou & Vanhoutte, 1988; Chen *et al.*, 1988; 1991; Nagao and Vanhoutte, 1992). However, the chemical nature of this factor is unknown. Komori & Vanhoutte (1990) suggested that the factor could be a labile metabolite of arachidonic acid. The epoxyeicosatrienoic acids, cytochrome P450 metabolites which could be synthesized in the vascular wall, activate K^+ channels in vascular smooth muscle cells (Gebremedhin *et al.*, 1992; Hu & Kim, 1993). Furthermore, some inhibitors of the cytochrome P450 pathway suppress the endothelium-dependent vasodilator responses which are resistant to inhibitors of nitric oxide and cyclo-oxygenase (Bauersachs *et al.*, 1995; Hecker *et al.*, 1995; Fulton *et al.*, 1995).

The aim of the present study was to investigate the role of the metabolism of arachidonic acid, especially the cytochrome P450 pathway, in the endothelium-dependent hyperpolarization evoked by acetylcholine in guinea-pig carotid arteries.

[1]Author for correspondence.

Methods

Electrophysiological experiments

Male Hartley guinea-pigs (300–400g) were anaesthetized with pentobarbitone (2.5 mg kg^{-1} i.p.) and the carotid arteries were dissected. Segments of artery (1 cm in length) were cleaned of adherent connective tissue and pinned down to the bottom of an organ bath (3 ml) continuously superfused with modified Krebs-Ringer bicarbonate solution (37°C, aerated with 95% O_2, 5% CO_2 gas mixture; pH 7.4) of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, calcium-disodium EDTA 0.026 and glucose 11.1. In most experiments, care was taken to preserve the endothelium as intact as possible; in some experiments, the endothelium was removed by a rapid infusion of saponin (1 mg ml^{-1}) in the lumen of the blood vessel. Transmembrane potentials were recorded with glass microelectrodes filled with KCl (3 M), with a tip resistance of 30 to 90 megohms. The microelectrode was mounted on a sliding micromanipulator (Leitz St Gallen, Switzerland). The potential recorded was amplified by means of a recording preamplifier (WPI (intra 767), New Haven, CT, USA) with capacitance-neutralization. The signal was monitored on an oscilloscope (3091 Nicolet, Madison, WI, USA) and continuously recorded on paper (Gould, Valley View, OH, USA) and on video recorder [TEAC XR310 (Tokyo, Japan)]; the latter allowed replay for further analysis. Impalements were not accepted as valid unless they were signalled by a sudden change in voltage, and were maintained for at least 3 min; at that point the membrane potential had stabilized. The impalements were performed from the intimal or adventitial side. The incubation time with the various inhibitors studied was at least 30 min.

Drugs

Acetylcholine, atropine, clotrimazole, indomethacin, 8-methoxypsoralen (methoxsalen), metyrapone, N$^{\omega}$-nitro-L-arginine (L=NOARG), 17-octadecynoic acid (17-ODYA), quinacrine, saponin substance P (Sigma La Verpillère, France); eicosatetraynoic acid (ETYA) (Cayman chemical company, Ann Arbor, MI, USA); SKF525a (β-diethylaminoethyl-diphenylpropyl-acetate HCL; Biomol research laboratories, Plymouth, PA, USA). All concentrations are expressed in molar concentrations (M) in the perfusate. ETYA and SKF525a (10 μM) were dissolved in dimethyl sulphoxide (DMSO) 0.1%, SKF525a (100 μM) and metyrapone were dissolved in DMSO 1%, indomethacin was dissolved in an equimolar concentration of Na_2CO_3, clotrimazole in chloroform 1%, 17-ODYA in ethanol 0.5% and methoxsalen in propylene glycol 1%. The other drugs were dissolved in distilled water.

Statistics

Data are shown as mean ± s.e. mean; *n* indicates the number of cells in which membrane potential was recorded. Statistical analysis was performed with Student's *t* test for unpaired observations. Differences were considered to be statistically significant when *P* was less than 0.05.

Results

The membrane potential of guinea-pig carotid artery smooth muscle cells was measured with microelectrode impalements from the adventitial or from the intimal side of the artery. No significant differences were observed between the resting membrane potentials measured by each route (adventitial side: -51.0 ± 2.7 mV, $n=9$; intimal side: -53.8 ± 3.4 mV, $n=8$).

In arteries with endothelium, substance P (100 nM) induced a transient hyperpolarization if the impalements were performed from the intimal side. However, the hyperpolarization was not observed or was greatly reduced in the case of adventitial penetration (Table 1). Acetylcholine (1 μM) produced a hyperpolarization of smooth muscle cells of arteries with endothelium, which was comparable both during intimal and adventitial penetration (Table 1).

In the presence of both L-NOARG (100 μM) and indomethacin (5 μM) (membrane potential; adventitial side: -57.8 ± 1.4 mV, $n=43$; intimal side: -52.9 ± 1.3 mV, $n=12$), substance P (100 nM) induced a transient hyperpolarization during impalement from the intimal side and acetylcholine (1 μM) during impalements from both the intimal and the adventitial side. The hyperpolarizations produced by these two mediators were not significantly affected by the inhibitors of the cyclo-oxygenase and nitric oxide synthase (Table 1, Figure 1). The hyperpolarizations induced by the two agonists were not observed when the endothelium had been removed (Figure 1).

In arteries with endothelium, in the presence of an elevated potassium concentration in the superfusing solution ($[K]_o = 35$ mM), the membrane potential was depolarized significantly to

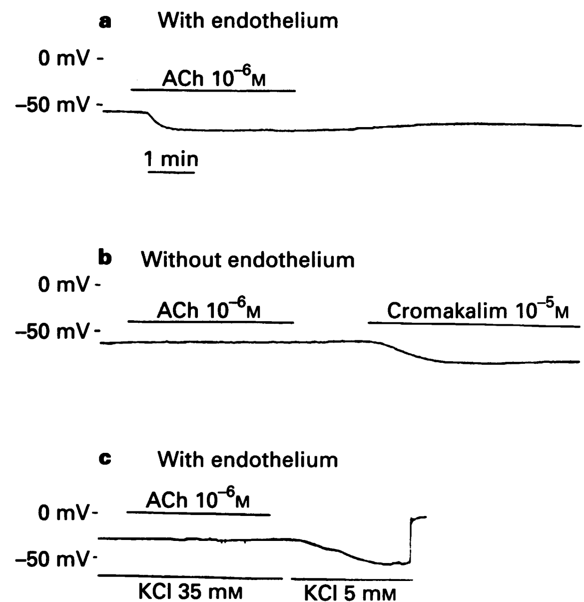

Figure 1 Endothelium-dependent hyperpolarization induced by acetylcholine (ACh: 1 μM) and substance P (SP : 100 nM) in guinea-pig isolated arteries. Indomethacin (5 μM) and L-NOARG (100 μM) were present throughout. (a) Guinea-pig carotid artery with endothelium: acetylcholine induced a hyperpolarization (membrane potential before acetylcholine infusion: -59 mV; membrane potential at the maximum of hyperpolarization: -78 mV). (b) Guinea-pig carotid artery without endothelium: acetylcholine did not influence the smooth muscle membrane (membrane potential before and after acetylcholine infusion: -64 mV). However, cromakalim (10 μM) induced a hyperpolarization (membrane potential at the maximum of hyperpolarization: -85 mV). (c) Guinea-pig carotid artery with endothelium, in the presence of elevated K^+ concentration (35 mM): Acetylcholine did not influence the smooth muscle membrane potential (membrane potential before and after acetylcholine infusion: -28 mV). Repolarization after restoration of control K^+ concentration (5 mM): -52 mV.

Table 1 Effect of substance P (100 nM) and acetylcholine (1 μM) on the membrane potential of the vascular smooth muscle of the guinea-pig artery

	Hyperpolarization (mV)	
	Substance P	*Acetylcholine*
Intimal side		
Control	15.7 ± 2.0 (n=3)	15.0 ± 1.9 (n=5)
L-NOARG + indomethacin	16.8 ± 2.2 (n=8)	13.5 ± 3.0 (n=4)
Adventitial side		
Control	0.3 ± 0.3 (n=3)	14.3 ± 2.8 (n=6)
L-NOARG + indomethacin	4.3 ± 2.0 (n=9)	15.1 ± 1.1 (n=35)

Values are means ± s.e.mean; *n*, number of cells in which membrane potential was recorded.

−32.8 ± 1.9 mV ($n = 9$) and substance P or acetylcholine did not produce a detectable change in membrane potential ($n = 3$ for each agonist, Figure 1).

Effect of cytochrome P450 inhibitors on acetylcholine-induced hyperpolarization

The effects of cytochrome P450 inhibitors on agonist-induced hyperpolarization of guinea-pig carotid artery were studied with acetylcholine (1 μM) used as an agonist. The vascular smooth muscle cell impalements were performed from the adventitial side, to avoid any impalements of the endothelial cells. The solvents used (DMSO, 1%, chloroform, 1%, ethanol, 0.5% and propylene, 1%) did not affect significantly either the basal membrane potential or the hyperpolarization induced by acetylcholine (Table 2). SKF525a (10 and 100 μM), metyrapone (100 μM), clotrimazole (100 μM), 17-ODYA (5 μM), methoxsalen (10 μM), quinacrine (10 μM) or ETYA (50 μM) did not modify the membrane potential and did not influence the acetylcholine-induced endothelium-dependent hyperpolarization (Figures 2 and 3, Table 2). The hyperpolarization induced by acetylcholine was abolished by atropine (100 nM) (Table 2).

Discussion

The present studies confirm previous findings in the guinea-pig carotid artery. The endothelium-dependent hyperpolarization evoked by acetylcholine and substance P in this tissue persists in the presence of inhibitors of nitric oxide synthase and cyclooxygenase (Chen *et al.*, 1991; Suzuki *et al.*, 1992). The hyperpolarizations induced by substance P are observed only if the impalements are performed from the intimal side of the artery, while those induced by acetylcholine are observed from both the adventitial and the intimal side (Zhang *et al.*, 1994). When impalements are performed from the adventitial side, the absence of effect of substance P could be due to an immediate degradation of this peptide by peptidases localized in the vessel wall, or to physical factors that render difficult the passage of this peptide through the vessel wall. Furthermore, the present experiments confirm the involvement of a potassium conductance in the hyperpolarization induced by acetylcholine or substance P, as elevating the extracellular potassium concentration, fully blocked the changes in membrane potential induced by these inhibitors (Chen *et al.*, 1991; Suzuki *et al.*, 1992).

Bioassay studies in canine coronary arteries have demonstrated that endothelium-dependent hyperpolarization is mediated by a diffusible substance (Félétou & Vanhoutte, 1988). Although the nature of this endothelium-derived hyperpolarizing factor has not been defined, it has been suggested that EDHF could be a cytochrome P450-derived metabolite of arachidonic acid (Komori & Vanhoutte, 1990). Indeed, some

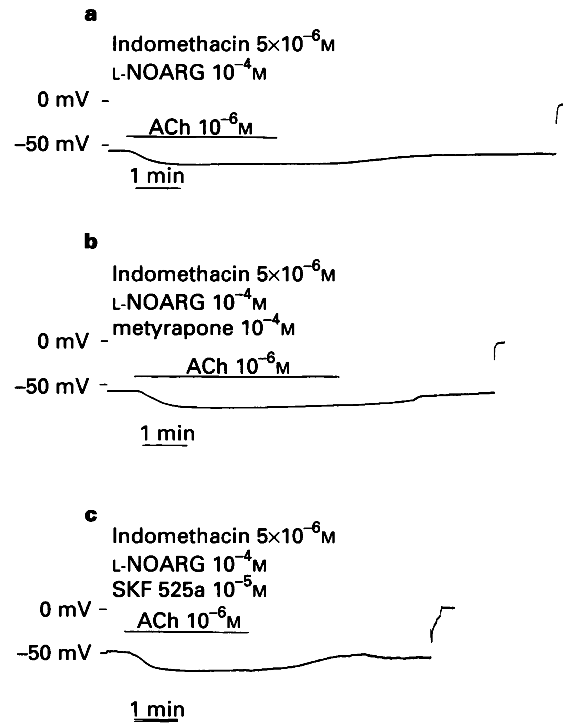

Figure 2 Metyrapone (100 μM), SKF525a (10 μM) and acetylcholine (ACh, 1 μM)-induced hyperpolarization in guinea-pig carotid arteries with endothelium. Indomethacin (5 μM) and L-NOARG (100 μM) were present throughout: (a) control: membrane potential before acetylcholine infusion: −57 mV; membrane potential at the maximum of hyperpolarization: −72 mV. (b) Metyrapone (100 μM). Membrane potential before acetylcholine infusion: −57 mV; membrane potential at the maximum of hyperpolarization: −76 mV. (c) *SKF525a* (10 μM). Membrane potential before acetylcholine infusion: −49 mV; membrane potential at the maximum of hyperpolarization: −71 mV.

Table 2 Effect of inhibitors of cytochrome P450, or their solvents and atropine, in presence of L-NOARG (100 μM) and indomethacin (5 μM), on hyperpolarization produced by acetylcholine (1 μM) in the carotid artery of the guinea-pig

	Membrane potential (mV)		
	Before acetylcholine	*After acetylcholine*	n
Control	−57.6 ± 1.6	−72.3 ± 1.8*	35
DMSO, 1%	−55.4 ± 1.2	−72.2 ± 1.8*	17
Ethanol, 0.5%	−56.0 ± 2.5	−73.5 ± 1.2*	4
Chloroform, 1%	−57.8 ± 2.2	−72.0 ± 1.5*	4
Propylene glycol, 1%	−58.1 ± 1.7	−75.9 ± 1.4*	8
SKF 525a, 10 μM	−54.6 ± 3.7	−68.5 ± 2.9*	8
SKF 525a, 100 μM	−56.0 ± 4.4	−71.3 ± 7.9*	3
Metyrapone, 100 μM	−58.6 ± 1.5	−71.7 ± 2.6*	10
Clotrimazole, 100 μM	−55.5 ± 2.4	−69.0 ± 1.8*	4
17-ODYA, 5 μM	−60.8 ± 2.8	−77.3 ± 1.4*	6
Methoxsalen, 10 μM	−54.0 ± 2.4	−70.4 ± 1.4*	9
Quinacrine, 10 μM	−56.6 ± 4.7	−69.4 ± 3.6*	5
ETYA, 50 μM	−54.0 ± 2.4	−69.0 ± 1.1*	6
Atropine, 100 nM	−65.0	−65.0	2

Values are means ± s.e.mean; *n*, number of cells in which membrane potential was recorded. *Indicates that acetylcholine induced a statistically significant change in membrane potential.

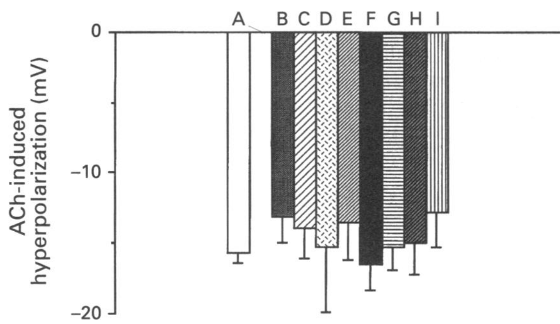

Figure 3 Inhibitors of arachidonic acid metabolism and hyperpolarizations elicited by acetylcholine (ACh, 1 μM) in guinea-pig carotid arteries. Experiments performed in the presence in L-NOARG (100 μM) and indomethacin (5 μM). (A) control; (B) metyrapone (10^{-4}M); (C) SKF525a (10^{-5}M); (D) SKF525a (10^{-4}M); (E) clotrimazole (10^{-4}M); (F) 17-ODYA (5×10^{-6}M); (G) methoxsalen (10^{-5}M); (H) ETYA (10^{-5}M); (I) quinacrine (10^{-5}M). These inhibitors did not significantly affect the endothelium-dependent hyperpolarization to acetylcholine. Values are mean $\pm$ s.e.mean. Metyrapone ($n=10$), SKF525a (10 μM, $n=8$; 100 μM, $n=3$), clotrimazole ($n=4$), 17-ODYA ($n=6$), methoxsalen ($n=9$), ETYA ($n=6$), quinacrine ($n=5$). For the sake of clarity, all the controls in their respective solvents have been pooled ($n=68$).

epoxyeicosatrienoic acids and especially the 11,12-epoxyeicosatrienoic acid, which may be synthesized by the endothelium, stimulate hyperpolarization of vascular smooth muscle (Grebremedhin *et al.*, 1992; Hu & Kim, 1993). Furthermore, in some vascular beds, cytochrome P450 inhibitors inhibit endothelium-dependent vasorelaxation resistant to cyclo-oxygenase and nitric oxide synthase blockade, although in these studies, the membrane potential was not measured (Hecker *et al.*, 1994; Bauersachs *et al.*, 1994; Fulton *et al.*, 1995).

In the present study, different cytochrome P450 inhibitors (SKF525a, metyrapone, clotrimazole, 17-ODYA and methoxsalen) did not inhibit the hyperpolarization produced by acetylcholine, although the concentrations studied were equivalent to or even higher than that used by other authors to block endothelium-dependent relaxations (Hecker *et al.*, 1994; Bauersachs *et al.*, 1994; Oyakan *et al.*, 1994; Weintraub *et al.*, 1994; Fulton *et al.*, 1995). These results were confirmed by the absence of inhibitory effect of ETYA, a non specific inhibitor of lipoxygenases/cyclo-oxygenases/cytochrome P450 mono-oxygenases and quinacrine and an inhibitor of phospholipase A_2. However, inhibition of the endothelium-dependent hyperpolarization induced by acetylcholine was obtained with the muscarinic antagonist, atropine.

Taken in conjunction, these results do not support the hypothesis that a metabolite of arachidonic acid is involved in endothelium-derived hyperpolarization produced by acetylcholine in the carotid artery of the guinea-pig. These discrepancies with the conclusion reached by Hecker *et al.* (1994) and Bauersachs *et al.* (1994) may be explained by differences between the species and/or the vascular beds studied. Alternatively, endothelium-dependent relaxation of vascular smooth muscle may be produced by several other mechanisms than hyperpolarization. Finally, some cytochrome P450 inhibitors used could be unspecific. Indeed, SKF525a inhibits the relaxation produced by drugs, which induce endothelium-independent relaxations, such as isoprenaline and diazoxide (Oyekan *et al.*, 1994). The poor aqueous solubility, the low potency and the nonspecific effects of these inhibitors may limit the use of these agents for probing the potential role of the metabolism of arachidonic acid by the cytochrome P450 mono-oxygenases (Harder *et al.*, 1995).

This work demonstrates that in guinea-pig carotid arteries, several inhibitors of cytochrome P450 do not inhibit the hyperpolarization produced by acetylcholine.

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