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Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig

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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 from the adventitial side with intracellular glass microelectrodes filled with KCl (30-80 MΩ).

2 Acetylcholine (1 μM) in the presence of inhibitors of nitric oxide synthase, (N^{ω}-nitro-L-arginine (L-NOARG) 100 μM) and cyclo-oxygenase, (indomethacin 5 μM) induced an endothelium-dependent hyperpolarization (-18.9 ± 1.6 mV, $n=15$).

3 In the presence of these two inhibitors, S-nitroso-L-glutathione (10 μM), sodium nitroprusside (10 μM), 3-morpholinosydnonimine (SIN-1, 10 μM) and iloprost (0.1 μM) induced endothelium-independent hyperpolarizations of the smooth muscle cells (respectively: -16.0 ± 2.3, -16.3 ± 3.4, -12.8 ± 2.0 and -14.5 ± 1.5 mV, $n=4-6$).

4 The addition of glibenclamide (1 μM) did not influence the acetylcholine-induced L-NOARG/indomethacin-resistant hyperpolarization (-18.0 ± 1.8 mV, $n=10$). In contrast, the responses induced by S-nitroso-L-glutathione, sodium nitroprusside, SIN-1 and iloprost were abolished (changes in membrane potential: -0.8 ± 1.1, 1.3 ± 3.9, 4.5 ± 4.6 and 0.3 ± 0.8 mV respectively, $n=4-5$).

5 In the presence of NO synthase and cyclo-oxygenase inhibitors, charybdotoxin (0.1 μM) or apamin (0.5 μM) did not influence the hyperpolarization produced by acetylcholine. However, in the presence of the combination of charybdotoxin and apamin, the acetylcholine-induced L-NOARG/indomethacin-resistant hyperpolarization was converted to a depolarization (4.4 ± 1.2 mV, $n=20$) while the endothelium-independent hyperpolarizations induced by S-nitroso-L-glutathione, sodium nitroprusside, SIN-1 and iloprost were not affected significantly (respectively: -20.4 ± 3.4, -22.5 ± 4.9, -14.5 ± 4.7 and -14.5 ± 0.5 mV, $n=4-5$).

6 In the presence of the combination of charybdotoxin and apamin and in the absence of L-NOARG and indomethacin, acetylcholine induced a hyperpolarization (-19.5 ± 3.7 mV, $n=4$). This hyperpolarization induced by acetylcholine was not affected by the addition of indomethacin (-18.3 ± 4.6 mV, $n=3$). In the presence of the combination of charybdotoxin, apamin and L-NOARG (in the absence of indomethacin), acetylcholine, in 5 out of 7 vessels, still produced hyperpolarization which was not significantly smaller (-9.1 ± 5.6 mV, $n=7$) than the one observed in the absence of L-NOARG.

7 These findings suggest that, in the guinea-pig isolated carotid artery, the endothelium-independent hyperpolarizations induced by NO donors and iloprost involve the opening of K_{ATP} channels while the acetylcholine-induced endothelium-dependent hyperpolarization (resistant to the inhibition of NO-synthase and cyclo-oxygenase) involves the opening of Ca^{2+}-activated potassium channel(s). Furthermore, in this tissue, acetylcholine induces the simultaneous release of various factors from endothelial origin: hyperpolarizing factors (NO, endothelium derived hyperpolarizing factor (EDHF) and prostaglandins) and possibly a depolarizing factor.

Keywords: Endothelium-derived hyperpolarizing factor; endothelium; smooth muscle; hyperpolarization; potassium channels; electrophysiology; acetylcholine; charybdotoxin; apamin; nitric oxide; prostacyclin

Introduction

Endothelium-derived relaxing factor (EDRF; identified as NO or a closely related substance (Palmer *et al.*, 1987; Myers *et al.*, 1990) and prostacyclin are relaxing substances released from endothelial cells in response to various stimuli including acetylcholine (Furchgott & Zawadzki 1980; De Mey & Vanhoutte 1981; for review Furchgott & Vanhoutte, 1989). In addition, endothelial cells release a yet unidentified endothelium-derived hyperpolarizing factor (EDHF) which, in vascular smooth muscle, causes membrane hyperpolarization by opening K^+ channels (Félétou & Vanhoutte, 1988; Chen *et al.*, 1988; 1991; Nagao & Vanhoutte, 1992). However EDRF/NO and NO donors which evoke relaxation through the activation of soluble guanylate cyclase (Rapoport & Murad, 1983) also produce smooth muscle cell hyperpolarization in some blood vessels (Ito *et al.*, 1980; Tare *et al.*, 1990; Murphy & Brayden, 1995). NO activates potassium channels through a cyclic GMP-dependent protein kinase (Robertson *et al.*, 1993; Taniguchi *et al.*, 1993) and, in rabbit aortic smooth muscle cells in culture, it also directly opens a calcium-dependent (charybdotoxin-sensitive) potassium conductance (Bolotina *et al.*, 1994). Endogenous vasoactive prostanoids could also mediate an endothelium-dependent hyperpolarization since the synthetic analogue of prostacyclin, methyl-prostacyclin (iloprost), induces hyperpolarization in guinea-pig coronary arteries (Parkington *et al.*, 1993). Vascular smooth muscle cells express different types of potassium channels (for review see Kuriyama *et al.*, 1995). ATP-sensitive (K_{ATP}) channels are blocked by glibenclamide and large conductance Ca^{2+}-activated (BK_{Ca}) channels by low concentrations of tetraethylammonium (TEA) or by charybdotoxin. An apamin-sensitive small-conductance Ca^{2+}-activated (SK_{Ca}) channel may also be expressed in vascular smooth muscle cells. In rabbit mesenteric arteries, apamin inhibits the endothelium-dependent hyperpolarizations to acetylcholine (Murphy & Brayden, 1995).

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The aim of the present experiments was to record the membrane potential of smooth muscle cells in the carotid artery of the guinea-pig and to study (1) the effects of various NO donors, of the synthetic analogue of prostacyclin, iloprost, and of the endogenous endothelium-derived factors released by acetylcholine and (2) to characterize the K^+ channels involved in these hyperpolarizations.

Methods

Electrophysiological experiments

Male Hartley guinea-pigs (300–400 g) were anaesthetized with pentobarbitone (2.5 mg kg^{-1}, intraperitonealy) and the carotid arteries were dissected free. Segments of artery (1 cm in length) were cleaned of adherent connective tissue and pinned down to the bottom of an organ chamber (3 ml) continuously superfused with modified Krebs-Ringer bicarbonate solution (37°C, aerated with a 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 destroyed by a rapid infusion of saponin (1 mg ml^{-1}) into the lumen of the blood vessel (Corriu *et al.*, 1996). Endothelial cell removal was considered to be successful when the hyperpolarization induced by acetylcholine did not exceed 5 mV. 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) with capacitance neutralization. The signal was monitored on an oscilloscope (3091 Nicolet (Madison, WI)) and continuously recorded on paper (Gould, Valley View, OH) 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 adventitial side. The incubation time with the various inhibitors studied was at least 20 min. Acetylcholine was infused for no longer than 5 min, to avoid desensitization of the preparation.

Drugs

Acetylcholine chloride; saponin; indomethacin; N^{ω}-nitro-L-arginine (L-NOARG), and sodium nitroprusside (Sigma, La Verpillère, France); charybdotoxin, apamin and scillatoxin (Latoxan, Rosans, France); glibenclamide (Boeringer, Mannheim, Germany); S-nitroso-L-glutathione (Alexis corporation, Läufelfingen, Switzerland); iloprost (Schering, Berlin, Germany); 3-morpholinosydnonimine (SIN-1) was synthetized in our laboratory (Servier, Suresnes, France). Glibenclamide was dissolved in dimethylsulphoxide (DMSO) 1%; indomethacin was dissolved in an equimolar concentration of Na_2CO_3. The other drugs were dissolved in distilled water. S-nitroso-L- glutathione was dissolved just before administration.

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. To compare data obtained from different groups a one way analysis of variance was used. When a significant interaction was observed ($P<0.05$), a complementary analysis was made (Newman-Keul's test) to identify differences between groups. Differences were considered to be statistically significant when *P* was less than 0.05.

Results

Acetylcholine (1 μM) hyperpolarizes the membrane of vascular smooth muscle cells of the guinea-pig carotid arteries (Tables 1 and 4). In previous work it has been demonstrated that acetylcholine-induced hyperpolarization depends on the presence of the endothelium and is abolished by atropine (100 nM) or by elevated potassium concentration ($[K]_0 = 35$ mM) (Corriu *et al.*, 1996).

In guinea-pig carotid artery smooth muscle cells, the resting membrane potential observed in the presence of inhibitors of NO synthase (L-NOARG, 100 μM) and cyclo-oxygenase (indomethacin, 5 μM) averaged −58.4 mV (Table 3). In the presence of these two inhibitors, acetylcholine induced hyperpolarization of the guinea-pig carotid artery smooth muscle (Figure 1, Table 1). Perfusion of the preparation with NO donors, S-nitroso-L-glutathione (10 μM), sodium nitroprusside (10 μM) and SIN-1 (10 μM), as well as with the prostacyclin analogue iloprost (0.1 μM), hyperpolarized the smooth muscle cells (Figure 1, Tables 1 and 2). The hyperpolarizations induced by S-nitroso-L-glutathione, sodium nitroprusside and iloprost did not depend on the presence of the endothelial lining (Tables 1 and 2). In the absence of L-NOARG and indomethacin, S-nitroso-L-glutathione and sodium nitroprusside induced hyperpolarizations of arteries with endothelium (Table 4).

Effects of potassium channel inhibitors in the presence of L-NOARG (100 μM) and indomethacin (5 μM)

Glibenclamide (1 μM) induced a small depolarization (3.5 mV) observed both in arteries with and without endothelium (Table

Table 1 Changes in membrane potential of smooth muscle cells from guinea-pig isolated carotid artery: effect of potassium channel inhibitors on acetylcholine and iloprost-induced hyperpolarization[a]

	Hyperpolarization (mV) *Acetylcholine (1 μM)*		*Iloprost (0.1 μM)*	
Control (with endothelium)	−18.9 ± 1.6	(*n* = 15)	−14.5 ± 1.5	(*n* = 4)
+ Glibenclamide (1 μM)	−18.0 ± 1.8	(*n* = 10)	−0.3 ± 0.8**	(*n* = 4)
+ Charybdotoxin (0.1 μM)	−19.0 ± 3.2	(*n* = 7)		
+ Apamin (0.5 μM)	−21.2 ± 2.5	(*n* = 6)		
+ Scillatoxin (50 nM)	−20.2 ± 2.4	(*n* = 5)		
+ Charybdotoxin and apamin	+4.4 ± 1.2**	(*n* = 20)	−14.5 ± 0.5	(*n* = 4)
+ Charybdotoxin and scillatoxin	−2.1 ± 4.2**	(*n* = 8)		
Control (without endothelium)	−0.4 ± 1.0##	(*n* = 16)	−16.6 ± 1.9	(*n* = 6)
+ Charybdotoxin and apamin	−0.1 ± 0.4#	(*n* = 8)		

[a]Experiments were performed in presence of L-NOARG (100 μM) and indomethacin (5 μM). Values are shown as mean ± s.e.mean; *n* represents the number of cells in which membrane potential was recorded. Asterisks indicate a statistically significant difference induced by potassium channel blockers (**$P \leq 0.01$). Sharp signs indicate statistically significant difference between arteries with and without endothelium (#$P \leq 0.05$, ##$P \leq 0.01$).

3). The endothelium-dependent hyperpolarization induced by acetylcholine was not modified by the addition of this potassium channel inhibitor. However, the effects of S-nitroso-L-glutathione, sodium nitroprusside, SIN-1 and iloprost were completely abolished by glibenclamide (Figure 2, Tables 1 and 2).

Scillatoxin (50 nM), charybdotoxin (0.1 μM) or apamin (0.5 μM) induced no or minor changes in the resting membrane of the smooth muscle cells in arteries with or without endothelium (Table 3). These inhibitors did not modify the hyperpolarizations induced by either acetylcholine, S-nitroso-L-glutathione, sodium nitroprusside or SIN-1 (Tables 1 and 2).

The combination of the two inhibitors charybdotoxin (0.1 μM) and apamin (0.5 μM) induced a significant depolarization of the resting membrane potential of the smooth muscle cells in arteries with and without endothelium (Table 3). In the presence of charybdotoxin and apamin, the acetylcholine-induced L-NOARG/indomethacin-resistant hyperpolarization was converted to a depolarization (Figure 3, Table 1). However, the hyperpolarization induced by NO donors and iloprost was not modified (Figure 3, Tables 1 and 2). The combination of scillatoxin (50 nM), and charybdotoxin (0.1 μM) also significantly inhibited the acetylcholine-induced endothelium-dependent hyperpolarization (Table 1).

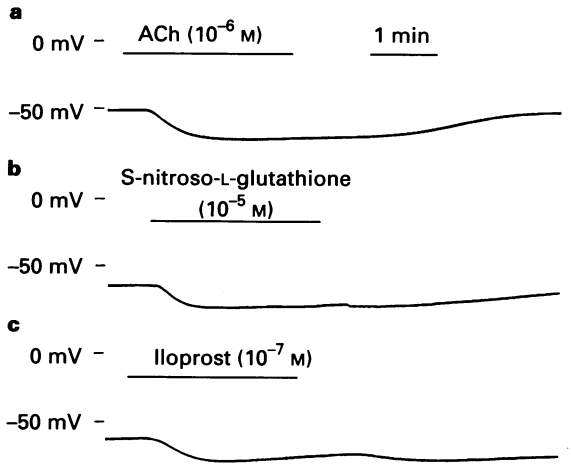

Figure 1 Effect of acetylcholine (ACh 1 μM, a), S-nitroso-L-glutathione (10 μM, b) and iloprost (0.1 μM, c) in guinea-pig carotid arteries with endothelium treated with indomethacin (5 μM) and L-NOARG (100 μM). Membrane potential before agonist infusion: −53, −65 and −64 mV and membrane potential at the nadir of hyperpolarization: −75, −83 and −80 mV, respectively, for ACh, S-nitroso-L-glutathione and iloprost.

Effects of indomethacin (5 μM) and/or L-NOARG (100 μM) in presence of potassium channel inhibitors charybdotoxin (0.1 μM) and apamin (0.5 μM)

In the presence of charybdotoxin (0.1 μM) plus apamin (0.5 μM) and in the absence of L-NOARG and indomethacin, acetylcholine (1 μM) induced hyperpolarization (Figure 4, Table 4). This hyperpolarization was not significantly affected by the addition of indomethacin (Figure 4, Table 4). In presence of L-NOARG (without indomethacin) acetylcholine induced hyperpolarization in 5 out of 7 preparations. However, the hyperpolarization recorded was not significantly smaller than the one observed in other conditions (Figure 4, Table 4).

In the presence of indomethacin, L-NOARG, charybdotoxin and apamin, acetylcholine induced a statistically significant depolarization (Figure 5, Table 4). This depolarization was not observed after removal of the endothelium (Figure 5, Table 4).

Discussion

Endothelium-dependent hyperpolarizations resistant to L-NOARG and indomethacin are mediated by a diffusible substance termed EDHF (Félétou & Vanhoutte, 1988; Chen *et al.*, 1991). However, the nature of this substance has not been identified. Depending on the vascular tissue or the species studied, prostacyclin (or iloprost) and NO (or NO donors) may or may not induce hyperpolarizations, indicating that in some instances they can act as endothelium-derived hyperpolarizing factors (Komori *et al.*, 1988; Tare *et al.*, 1990; Parkington *et al.*, 1993; Plane & Garland, 1993; Plane *et al.*, 1995; Murphy & Brayden, 1995). However, in most cases NO or/and prostacyclin cannot fully explain the acetylcholine-induced endothelium-dependent hyperpolarizations (for review: Cohen & Vanhoutte, 1995). In the carotid artery of the guinea-pig, NO donors and iloprost, a stable analogue of prostacyclin, induced hyperpolarizations sensitive to glibenclamide, indicating the activation of K_{ATP} channels which is in agreement with other studies (Jackson *et al.*, 1993; Parkington *et al.*, 1993; Miyoshi *et al.*, 1994; Plane *et al.*, 1995; Murphy & Brayden, 1995). However, in the rabbit, patch clamp experiments have shown that nitric oxide directly activates a BK_{Ca} conductance (Bolotina *et al.*, 1994). Whether this difference could be attributed to true species heterogeneity or to the differences in the techniques used is uncertain.

In the presence of NO synthase and cyclo-oxygenase inhibitors, acetylcholine-induced endothelium-dependent hyperpolarizations were insensitive to glibenclamide indicating that EDHF target does not involve the opening of K_{ATP}. Charybdotoxin (an inhibitor of BK_{Ca}), apamin, or scillatoxin (two inhibitors of SK_{Ca}) did not individually modify the am-

Table 2 Changes in membrane potential of smooth muscle cells from isolated guinea-pig carotid artery: effect of potassium channel inhibitors on S-nitroso-L-glutathione (SNOG), sodium nitroprusside (SNP) or 3-morpholinosydnonimine (SIN-1)-induced hyperpolarization[a]

	Hyperpolarization (mV)					
	SNOG (10 μM)		*SNP (10 μM)*		*SIN-1 (10 μM)*	
Control (with endothelium)	−16.0±2.3	(n=5)	−16.3±3.4	(n=6)	−12.8±2.0	(n=6)
+Glibenclamide (1 μM)	−0.8±1.1**	(n=5)	+1.3±3.9**	(n=4)	+4.5±4.6**	(n=4)
+Charybdotoxin (0.1 μM)	−17.3±3.3	(n=4)	−19.5±4.9	(n=4)	−17.0±3.2	(n=3)
+Apamin (0.5 μM)	−17.3±2.5	(n=6)	−17.0±3.0	(n=3)	−19.7±0.3	(n=3)
+Scillatoxin (50 nM)	−17.0±0.6	(n=3)	−15.7±2.3	(n=3)	−17.5±1.2	(n=4)
+Charybdotoxin and apamin	−20.4±3.4	(n=5)	−22.5±4.9	(n=4)	−14.5±4.7	(n=4)
Control (without endothelium)	−16	(n=2)	−11.8±1.8	(n=4)		

[a]Experiments were performed in presence of L-NOARG (100 μM) and indomethacin (5 μM). Values are shown as mean±s.e.mean; *n* indicates the number of cells in which membrane potential was recorded. Asterisks indicate a statistically significant difference induced by potassium channel blockers (**$P \leq 0.01$).

Table 3 Membrane potential of smooth muscle cells from guinea-pig isolated carotid artery: Effect of potassium channel inhibitors[a]

	Membrane potential (mV)	
	With endothelium	*Without endothelium*
Control	-58.4 ± 1.2 ($n=36$)	-54.3 ± 1.2 ($n=38$)
+ Glibenclamide (1 μM)	-54.9 ± 1.1* ($n=27$)	-50.72 ± 1.5 ($n=11$)
+ Charybdotoxin (0.1 μM)	-56.0 ± 1.8 ($n=19$)	-57.0 ± 1.4 ($n=11$)
+ Apamin (0.5 μM)	-54.0 ± 1.2* ($n=19$)	-53.3 ± 1.7 ($n=16$)
+ Scillatoxin (50 nM)	-56.3 ± 1.9 ($n=15$)	
+ Charybdotoxin and apamin	52.5 ± 1.6**($n=37$)	-48.4 ± 2.0* ($n=22$)
+ Charybdotoxin and scillatoxin	-50.9 ± 3.9* ($n=8$)	

[a]Experiments were performed in the presence of L-NOARG (100 μM) and indomethacin (5 μM). Values are shown as mean ± s.e.mean; n indicates the number of cells in which membrane potential was recorded. Asterisks indicate a statistically significant difference induced by potassium channel blockers (*$P \leq 0.05$; **$P \leq 0.01$).

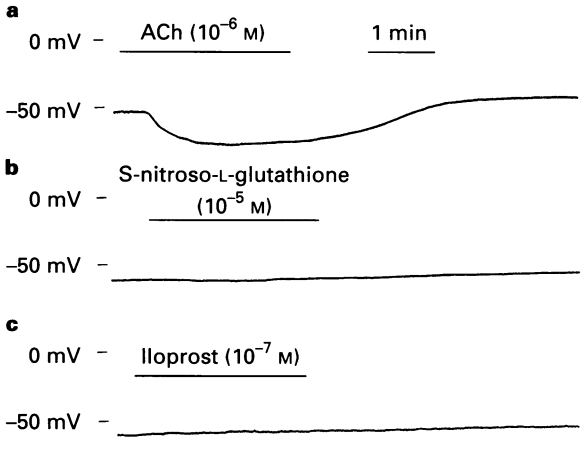

Figure 2 Effect of acetylcholine (ACh 1 μM, a), S-nitroso-L-glutathione (10 μM, b) and iloprost (0.1 μM, c) in guinea-pig carotid arteries with endothelium treated with the combination of indomethacin (5 μM), L-NOARG (100 μM) and glibenclamide (1 μM). Membrane potential before agonist infusion: −54, −62 and −59 mV and membrane potential at the nadir of hyperpolarization: −80, −63 and −59 mV, respectively, for ACh, S-nitroso-L-glutathione and iloprost.

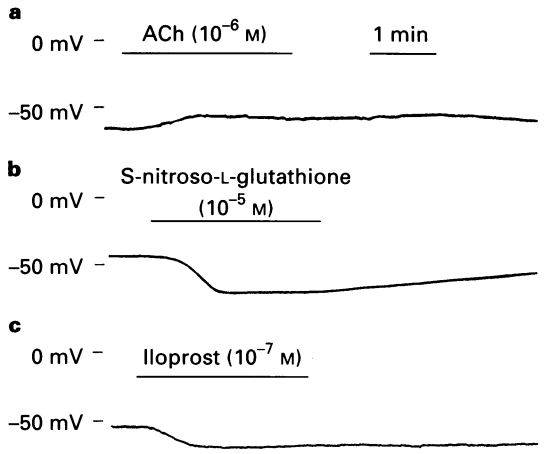

Figure 3 Effect of acetylcholine (ACh: 1 μM, a), S-nitroso-L-glutathione (10 μM, b) and iloprost (0.1 μM, c) in guinea-pig carotid arteries with endothelium treated with indomethacin (5 μM) and L-NOARG (100 μM) and the combination of charybdotoxin (0.1 μM) plus apamin (0.5 μM). Membrane potential before agonist infusion: −65, −44, −55 mV and membrane potential at the maximum effect: −56, −71 and −72 mV, respectively, for ACh, S-nitroso-L-glutathione and iloprost.

plitude of the hyperpolarizations induced by the muscarinic agonist. However, the combination of two inhibitors, charybdotoxin plus apamin, or charybdotoxin plus scillatoxin, blocked the effect of acetylcholine. The combination of the two inhibitors significantly depolarized the resting membrane potential in vessels with and without endothelium. This suggests a direct blockade of Ca^{2+}-activated potassium channels at the smooth muscle cell level. Presuming that the combination of these two inhibitors does not affect the synthesis or the release of EDHF from endothelial cells, the effect of EDHF could involve the activation of Ca^{2+}-dependent potassium conductances. However, as the combination of two potassium channel antagonists was needed, it remains to be determined whether two Ca^{2+}-activated potassium channels (BK_{Ca} and SK_{Ca}) or a single population of Ca^{2+}-activated channel, not previously described, sensitive to the two toxins are/is activated by EDHF. These results confirm previous data showing that endothelium-dependent hyperpolarizations (or relaxations) resistant to the inhibition of NO synthase and cyclo-oxygenase are unaffected by glibenclamide, but could be sensitive to apamin or to charybdotoxin (Brayden 1990; Hwa *et al.*, 1994; Zygmunt *et al.*, 1994; Hatake *et al.*, 1995; Murphy & Brayden, 1995).

In the present study, the acetylcholine-induced hyperpolarization attributed to EDHF (e.g. resistant to nitric oxide synthase and cyclo-oxygenase inhibitors) was selectively inhibited by the combination of the two potassium channels inhibitor (charybdotoxin + apamin). Therefore, this treatment allowed the study of endothelium-dependent hyperpolarization induced by the release of factors other than EDHF. In the presence of the two potassium channel inhibitors, the addition of indomethacin (in the absence of L-NOARG) did not influence the hyperpolarization induced by acetylcholine, demonstrating that nitric oxide released by the cholinergic agonist produced the hyperpolarization. On the other hand, in the absence of indomethacin, but in the presence of the nitric oxide synthase inhibitor (and the two potassium channel inhibitors), acetylcholine again induced hyperpolarization, indicating that in the guinea-pig carotid artery, endothelial arachidonic acid metabolites through the cyclo-oxygenase pathway provoke hyperpolarization of the smooth muscle cells. In the carotid artery of the guinea-pig, nitric oxide and prostaglandins can be considered as EDHF(s) as has already been suggested in the coronary artery of the same species (Parkington *et al.*, 1993).

In the presence of the inhibitors of NO synthase and cyclo-oxygenase plus the combination of charybdotoxin and apamin, acetylcholine induced a depolarization which was endothelium-dependent. This endothelium-dependent depolarization may be due to the release of a depolarizing factor as has been suggested by Mombouli *et al.* (1995). Acetylcholine induces endothelium-dependent contraction by releasing arachidonic acid metabolites, possibly 20-hydroxyeicosatetraenoic acid (Escalante *et al.*, 1993; Ma *et al.*, 1993; Nishimura *et al.*,

Table 4 Changes in membrane potential of smooth muscle cells from guinea-pig isolated carotid artery (with endothelium) induced by acetylcholine (ACh), S-nitroso-L-glutathione (SNOG) or sodium nitroprusside (SNP): effects of L-NOARG, indomethacin, potassium channel inhibitors and their combination

	Hyperpolarization (mV)		
	ACh (1 μM)	*SNOG (10 μM)*	*SNP (10 μM)*
Control	−13.3±2.7 (*n*=7)	−9.1±1.5* (*n*=5)	−14.4±2.2 (*n*=5)
+L-NOARG (100 μM) and indomethacin (5 μM)	−18.9±1.6 (*n*=15)	−16.0±2.3 (*n*=5)	−16.3±3.4 (*n*=6)
+Charybdotoxin (0.1 μM) and apamin (0.5 μM)	−19.5±3.7 (*n*=4)		
+Charybdotoxin, apamin and indomethacin	−18.3±4.7 (*n*=3)		
+Charybdotoxin, apamin and L-NOARG	−9.1±5.6 (*n*=7)		
+Charybdotoxin, apamin indomethacin and L-NOARG	+4.4±1.2* (*n*=20)	−20.4±3.4 (*n*=5)	−22.5±4.9 (*n*=4)

Values are shown as mean±s.e.mean; *n* represents the number of cells in which membrane potential was recorded. Asterisks indicate a statistically significant difference when compared with the effect of agonist in presence of L-NOARG and indomethacin *$P \leq 0.05$. The changes in membrane potential induced by acetylcholine in the presence of charybdotoxin, apamin, indomethacin and L-NOARG were significantly different from that produced by acetylcholine in all other conditions (Newman Keul's test, $P \leq 0.05$).

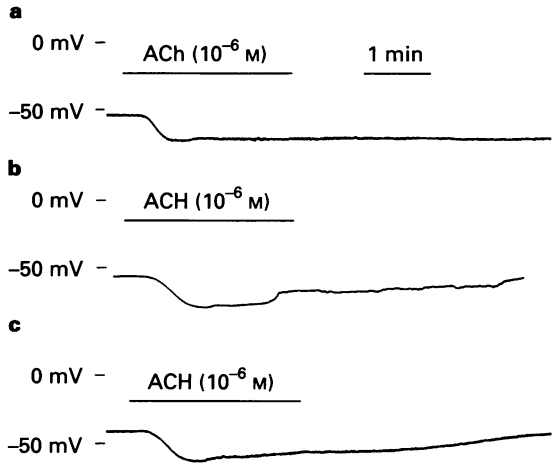

Figure 4 Effect of acetylcholine (ACh: 1 μM) in guinea-pig carotid arteries with endothelium treated with various inhibitors. (a) Charybdotoxin (0.1 μM) plus apamin (0.5 mM); (b) charybdotoxin (0.1 μM) plus apamin (0.5 μM) plus indomethacin (5 μM); (c) charybdotoxin (0.1 μM) plus apamin (0.5 μM) plus L-NOARG (100 μM). Membrane potential before ACh infusion: −54, −53 and −42 mV and membrane potential at the nadir of hyperpolarization: −72, −76 and −63 mV, respectively, for (a), (b) and (c).

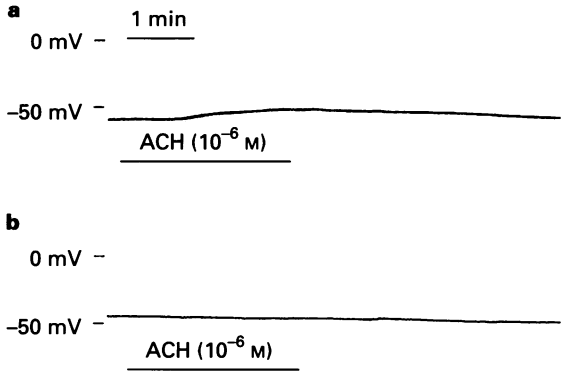

Figure 5 Effect of acetylcholine (ACh: 1 μM) in guinea-pig carotid arteries treated with the combination of charybdotoxin (0.1 μM), apamin (0.5 μM) indomethacin (5 μM) and L-NOARG (100 μM). (a) Vessel with endothelium; (b) vessel without endothelium. Membrane potential before ACh infusion: −60, and −43 mV and membrane potential at the maximum effect: −53 and −43 mV, respectively, for vessels with (a) and without (b) endothelium.

1995). The present study does not allow further speculation on the mechanism involved in the endothelium-dependent depolarization observed in the guinea-pig carotid artery.

In conclusion, the present findings demonstrate that in the presence of inhibitors of NO-synthase and cyclo-oxygenase, NO donors, iloprost and acetylcholine may hyperpolarize the cell membrane of smooth muscle cells of the isolated guinea-pig carotid artery. However, the hyperpolarization induced by NO donors and prostacyclin involves the opening of K_{ATP} channels, while the hyperpolarization provoked by acetylcholine (resistant to L-NOARG/indomethacin) involves Ca^{2+}-activated potassium channel(s). Furthermore, these results suggest that in this tissue, endothelial cells stimulated by acetylcholine release simultaneously various hyperpolarizing factors (NO, EDHF and prostaglandins) and possibly a depolarizing substance.

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