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Roles of calcium-activated and voltage-gated delayed rectifier potassium channels in endothelium-dependent vasorelaxation of the rabbit middle cerebral artery

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1 The cellular mechanism(s) of action of endothelium-derived vasodilator substances in the rabbit middle cerebral artery (RMCA) were investigated. Specifically, the subtypes of potassium channels involved in the effects of endothelium-derived relaxing factors (EDRFs) in acetylcholine (ACh)-induced endothelium-dependent vasorelaxation in this vessel were systematically compared.

2 In the endothelium-intact RMCA precontracted with histamine (3 μM), ACh induced a concentration-dependent vasorelaxation, which was sensitive to indomethacin (10 μM) or N^G-nitro-L-arginine (L-NOARG; 100 μM); pD_2 values 8.36 vs 7.40 and 6.38, $P<0.01$ for both, $n=6$ and abolished by a combination of both agents. ACh caused relaxation in the presence of high K^+ PSS (40 mM KCl), which was not affected by indomethacin, but abolished by L-NOARG and a combination of indomethacin and L-NOARG.

3 In the presence of indomethacin, relaxation to ACh in the endothelium-intact RMCA precontracted with histamine was unaffected by either glibenclamide (10 μM), an ATP-sensitive K^+ channel (K_{ATP}) blocker, 4-aminopyridine (4-AP, 1 mM) or dendrotoxin (DTX, 0.1 μM), delayed rectifier K^+ channel (K_V) blockers. However, relaxation responses to ACh were significantly inhibited by either LY83583 (10 μM) and 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 10 μM), guanylyl cyclase inhibitors, or charybdotoxin (CTX; 0.1 μM), iberiotoxin (ITX, 0.1 μM) and apamin (APA, 0.1 μM), large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) blocker and small conductance Ca^{2+} -activated K^+ channel (SK_{Ca}) blocker, respectively.

4 In the presence of L-NOARG, relaxation to ACh was unaffected by glibenclamide or the cytochrome P450 mono-oxygenase inhibitor, clotrimazole (1 μM), but was significantly inhibited by either 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22,536, 10 μM) and 2′,3′-dideoxyadenosine (2′,3′-DDA, 30 μM), adenylyl cyclase inhibitors, or 4-AP, DTX, CTX, ITX and APA.

5 In the endothelium-denuded RMCA precontracted with histamine, authentic NO-induced relaxation was unaffected by glibenclamide, 4-AP and DTX, but significantly reduced by ODQ, ITX and APA. Authentic prostaglandin I_2 (PGI_2)-induced relaxation was unaffected by glibenclamide, but significantly reduced by 2′,3′-DDA, 4-AP, DTX, ITX and APA. Forskolin-induced relaxation was significantly inhibited by high K^+, CTX and 4-AP.

6 These results indicate that: (1) in the RMCA the EDRFs released by ACh are NO and a prostanoid (presumably PGI_2), and there is no evidence for the release of a non-NO/PGI_2 endothelium-derived hyperpolarizing factor (EDHF), (2) K_{Ca} channels are involved in NO-mediated relaxation of the RMCA but both K_{Ca} and K_v channels are involved in PGI_2-mediated relaxation.

Keywords: Acetylcholine; nitric oxide; prostacyclin; guanylyl cyclase; adenylyl cyclase; calcium-activated potassium channels; voltage-gated delayed rectifier potassium channels; vasorelaxation; cerebral arteries

Introduction

Endothelium-dependent vasorelaxation is considered to be produced by at least three vasodilators: prostacyclin (PGI_2), endothelium-derived relaxing factor (EDRF) and endothelium-derived hyperpolarizing factor (EDHF; Gryglewski *et al*., 1986; Palmer *et al*., 1987; Parkington *et al*., 1993; Hammarström *et al*., 1995). The roles of PGI_2 and NO in mediating endothelium-dependent vasorelaxation are well established, although some debate exists as to the cellular mechanisms involved (Palmer *et al*., 1988; Lamontagne *et al*., 1992; Bolotina *et al*., 1994). PGI_2 is known to elevate intracellular levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Shaul *et al*., 1992; Tamaoki *et al*., 1993; Armstead, 1995) leading to smooth muscle relaxation by enhancing Ca^{2+} uptake into the sarcoplasmic reticulum and/or activation of K^+ channels (Siegel *et al*., 1989; Adeagbo & Malik, 1990; Minami *et al*., 1993; Parkington *et al*., 1995). NO activates guanylyl cyclase, elevates intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP), and enhances sarcoplasmic reticulum Ca^{2+} uptake (Moncada *et al*., 1991). However, NO has also been shown to enhance the activity of large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) either directly (Bolotina *et al*., 1994) or indirectly via cyclic GMP and cyclic GMP-dependent protein kinase (Moncada *et al*., 1991; Robertson *et al*., 1993).

In contrast, the identity and cellular mechanism(s) of action of EDHF are poorly defined (Garland *et al*., 1995; Waldron *et al*., 1996). Moreover, the contribution of EDHF to endothelium-dependent relaxation appears to depend on tissue source, species and agonist employed to induce vasorelaxation (Nagao & Vanhoutte, 1993; Garland *et al*., 1995; Waldron *et al*., 1996). The contribution of EDHF to endothelium-

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dependent relaxation in small cerebral resistance arteries still remains elusive and its contribution to ACh-induced endothelium-dependent vasorelaxation in the rabbit middle cerebral artery (RMCA) is controversial. For instance, Brayden (1990) suggested that hyperpolarization in RMCA during ACh treatment resulted from the release of EDHF, in which activation of K_{ATP} channels were involved. In contrast, Parsons *et al*. (1991) and Wellman and Bevan (1995) have shown that NO mediates the vast majority of the response to ACh and incubation with glibenclamide had no effect on ACh-induced relaxation in the same vessel. In addition, characterization of the K^+ channels involved in endothelium-dependent relaxation in RMCA have not been elucidated, although many studies have been carried out in peripheral arteries and suggest that the subtypes of K^+ channels involved in endothelium-derived vasodilators depend on tissue source and species (Schubert *et al*., 1996; 1997; Li *et al*., 1997; Zhao *et al*., 1997).

Given that several pathophysiological conditions are associated with impaired endothelium-dependent responses, including ischaemia, vasospasm and stroke (Faraci, 1993), it is essential that the mechanisms for endothelium-dependent vasodilatation in the cerebral vasculature be determined. Therefore, the present study was undertaken to investigate systematically the contributions of prostanoids, NO and EDHF to endothelium-dependent vasorelaxation in the rabbit middle cerebral resistance arteries (Baumbach & Heistad, 1983; Brayden, 1990). In the present study we showed that NO and a prostanoid (presumably PGI_2) but not EDHF contribute to endothelium-dependent vasorelaxation to ACh in RMCA. We have also performed experiments to investigate the subtypes of K^+ channels involved in the cellular mechanisms of action of endothelium-derived vasodilators in cerebral arteries: agents were employed to inhibit selectively the activity of different subtypes of K^+ channels, adenylyl cyclase, guanylyl cyclase and cytochrome P450 mono-oxygenase during ACh-induced endothelium-dependent vasorelaxation. Some of the data presented here have been previously published in abstract form (Dong *et al*., 1996).

Methods

Preparation of cerebral arteries

Adult male New Zealand white rabbits (2–2.5 kg) were anaesthetized with sodium pentobarbitone (240 mg kg^{-1}) and killed according to a research protocol consistent with the standards of the Canadian Council on Animal Care and approved by the local Animal Care Committee of The University of Calgary. Brains were rapidly removed and placed in cold physiological salt solution (PSS) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and dextrose 11.1. The pH of the solution after saturation with 95% O_2 + 5% CO_2 gas mixture was 7.4. Solutions of high K^+ PSS (40 mM KCl) were made by equimolar replacement of NaCl with KCl. Segments of middle cerebral artery (200–250 μm i.d.) were gently dissected from the surface of the brain and placed in fresh PSS. Adherent connective tissue was removed and a 2 mm segment of artery was then prepared for recording isometric force development in a wire myograph as previously described (Waldron & Garland, 1994). Briefly, two tungsten wires (40 μm diameter) were inserted through the lumen of the vessel and the tissue placed in a 10 ml myograph chamber containing gassed PSS. One wire was then attached to a force transducer and the other connected to a micrometer. In some experiments, endothelial cells were removed from the vessels by repeatedly passing a stainless steel cannula through the vessel lumen. Cannulae that had external diameters slightly larger than the lumenal diameter of the artery were used. Destruction of the endothelium was confirmed pharmacologically by loss of relaxation response to ACh (10 μM). After a 30 min equilibration period, arteries were stretched in a stepwise fashion to a resting tension of 2 mN (Wellman & Bevan, 1995). This was found to be the optimal preload for force development in these blood vessels in preliminary studies. Tissues were routinely allowed to equilibrate for 2 h before the start of the experiments. Isometric tension was recorded to a hard disc via an A/D converter (Axon Instruments, Foster City, U.S.A.) and analysed by commercial software (Axotape 2.0, Axon Instruments) running in a 486 IBM clone computer.

Experimental protocols

The first series of experiments was performed to determine the contributions of prostanoids, NO and EDHF to endothelium-dependent vasorelaxation in segments of RMCA. Arterial rings were precontracted with either histamine (3 μM), or high K^+ PSS (40 mM) and subsequently relaxed by ACh in half-log increments in concentration (0.001–30 μM). The tone developed by histamine or high K^+ PSS was similar (10.5±0.5 mN vs 10.2±0.8 mN, $P>0.05$, $n=10$). The second ACh concentration-response curves were always performed after 1 h of washing with PSS and blockers (indomethacin, 10 μM; N^G-nitro-L-arginine (L-NOARG, 100 μM); or a combination of both agents) were added to the tissues 30 min before the commencement of the second curve. Pretreatment with L-NOARG did not affect basal tone of the vessels, but it increased the sensitivity of the vessels to histamine. To rule out the possibility of functional antagonism, in the presence of L-NOARG, the concentration of histamine was adjusted to ensure a similar level of tone to control conditions (10.4±0.6 mN vs 10.8±0.4 mN, $P>0.05$, $n=6$). In a second series of experiments, to address the cellular mechanisms involved in vasodilatation to NO or a prostanoid, initial concentration-response curves for ACh were obtained in the presence of indomethacin or L-NOARG, and then compared to the extent of relaxation during a second exposure to ACh performed under similar conditions plus the presence of another blocker. In these experiments, the second concentration-response curves were performed with: (a) either 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22,536, 10 μM) or 2′,3′-dideoxyadenosine (2′,3′-DDA, 30 μM) to inhibit adenylyl cyclase, LY83583 (10 μM) or 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 10 μM) to inhibit guanylyl cyclase, (b) glibenclamide (10 μM), apamin (1 μM), iberiotoxin (ITX, 0.1 μM), charybdotoxin (CTX, 0.1 μM), 4-aminopyridine (4-AP, 1 mM) or dendrotoxin (DTX, 0.1 μM) to inhibit selectively the activity of ATP-sensitive K^+ (K_{ATP}), small conductance Ca^{2+}-activated K^+ (SK_{Ca}), large conductance Ca^{2+}-activated K^+ (BK_{Ca}), or voltage-dependent delayed rectifier K^+ (K_V) channels, respectively, or (c) clotrimazole (1 μM) to inhibit cytochrome P450 mono-oxygenase. Because pretreatment with CTX and 4-AP induced increase in the basal tone of the vessels, the concentration of histamine was then decreased after pretreatment with them in order to induce similar tone compared to that before pretreatment with these drugs. All other blockers used in the present study did not affect the basal level of tone in the RMCA. In most experiments, only two concentration-response curves were obtained for an individual tissue. However, when the agent employed was found to be without effect on endothelium-

dependent relaxation in RMCA, a third concentration-response curve to ACh was obtained after 1 h of washing and 30 min incubation with a combination of indomethacin and L-NOARG to serve as a positive control. In preliminary experiments, control curves to ACh were performed either in the absence or presence of indomethacin, L-NOARG or both agents to determine the effects of time and repeated exposure to ACh on the magnitude of the vasorelaxation. No significant difference was identified during three sequential concentration-response curves to ACh under these conditions.

Drugs

The following drugs were used: histamine dihydrochloride, acetylcholine chloride, indomethacin, glibenclamide, apamin, charybdotoxin, 4-AP, 2′,3′-DDA, prostacyclin and clotrimazole were purchased from Sigma Chemical Company (St. Louis, USA). Iberiotoxin, N^G-nitro-L-arginine, dendrotoxin and SQ 22,538 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine) were purchased from Research Biochemicals, Inc. (Natick, U.S.A). ODQ was purchased from Tocris Cookson Inc. (St. Louis, USA). LY83583 (6-anilinoquinoline-5,8-quinone) was a gift from Lilly Research Laboratories (Indianapolis, U.S.A.). Stock solutions of clotrimazole (1 mM), indomethacin (1 mM) and glibenclamide (10 mM) were prepared in ethanol, 4% (w/v) $NaHCO_3$ and dimethylsulphoxide, respectively. All other compounds dissolved freely in distilled water. At the final concentrations employed (0.1%), none of the solvents used had any effect on the vessels during preliminary experiments. Nitric oxide solution was made as described previously (Dong *et al*., 1997).

Statistical analysis

Vasorelaxant responses were measured as percentage inhibition of contraction induced by histamine or high K^+ PSS. pD_2 values were determined as the negative log molar concentration of ACh which caused 50% of the maximal vasorelaxant effect. All data were expressed as means ± s.e.mean and compared by Student's *t* test for paired data and were considered significant at $P<0.05$. In all experiments *n* equals the number of animals from which RMCA were removed.

Results

Contributions of NO, prostanoid and EDHF to ACh-induced endothelium-dependent vasorelaxation in RMCA

In the endothelium-intact RMCA precontracted with histamine (3 μM), ACh induced a concentration-dependent relaxation (Figure 1). This ACh-induced vasorelaxation was completely abolished by removal of the endothelium ($n=5$, data not shown). The cyclo-oxygenase inhibitor, indomethacin (10 μM) significantly inhibited the concentration-response curves for ACh. Both the pD_2 value and the maximal relaxation to ACh after treatment with indomethacin were decreased (8.36 ± 0.14 vs 7.40 ± 0.11, $P<0.01$ and 105 ± 1% vs 93 ± 1%, $P<0.05$, $n=6$ for both). The NO synthase inhibitor, L-NOARG (100 μM) produced a larger inhibition of the concentration-response curves for ACh (Figure 1), with both the pD_2 value and the maximal relaxation decreased

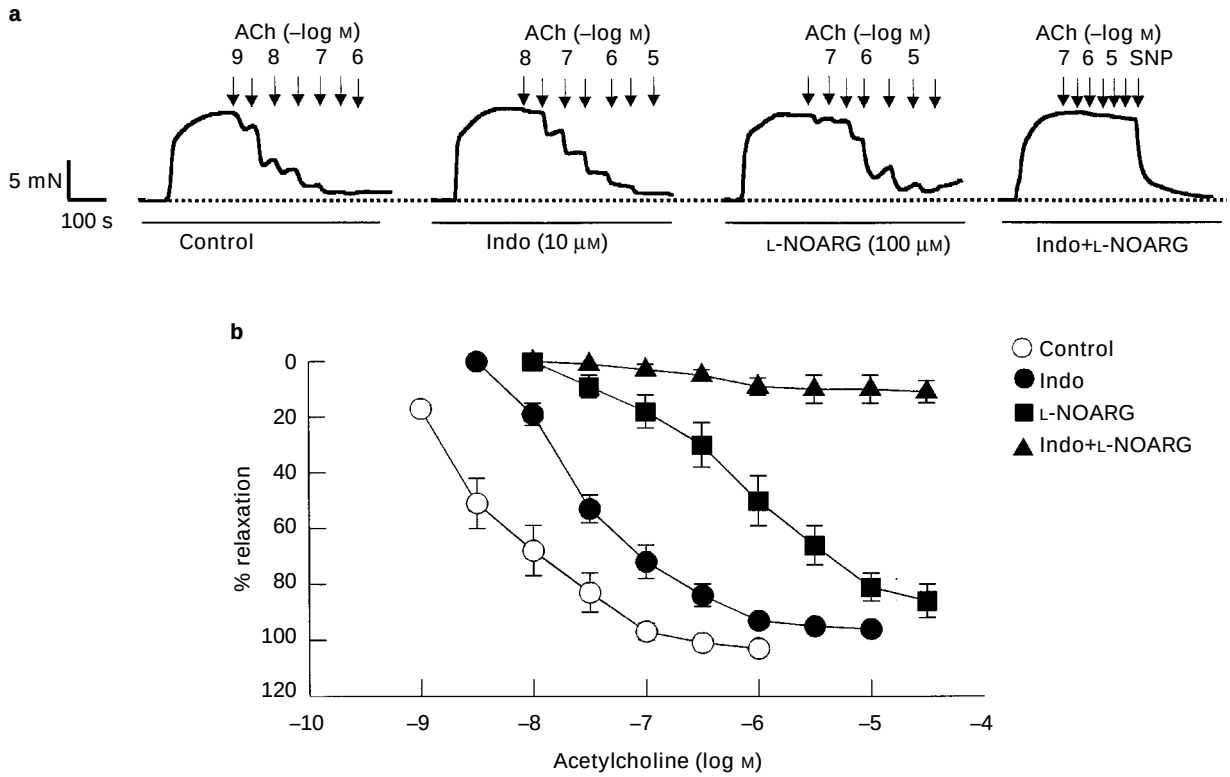

Figure 1 (a) Representative recordings of acetylcholine (ACh)- and sodium nitroprusside (SNP, 10 μM)-induced relaxation of the endothelium-intact rabbit middle cerebral artery precontracted with histamine (1–3 μM) in the absence and the presence of indomethacin (Indo, 10 μM), N^G-nitro-L-arginine (L-NOARG, 100 μM) or indomethacin plus N^G-nitro-L-arginine. Acetylcholine was added in half-log concentration increments at the arrows, although the numerical values of the half-log concentrations are omitted for clarity. (b) Mean concentration-response curves for acetylcholine in the absence and presence of indomethacin (10 μM), N^G-nitro-L-arginine (100 μM) or indomethacin plus N^G-nitro-L-arginine. Points are the mean from 6 separate experiments; vertical lines show s.e.mean.

(8.41 ± 0.10 vs 6.38 ± 0.18, $P<0.01$ and $103 \pm 1\%$ vs $86 \pm 5\%$, $P<0.05$, $n=6$ for both). However, when vessels were treated with a combination of indomethacin and L-NOARG, the ACh-induced vasorelaxation was completely blocked and the maximal relaxation to ACh was decreased from $104 \pm 1\%$ in the absence of both inhibitors to $9 \pm 4\%$ in the presence of both inhibitors ($P<0.01$, $n=6$). In tissues contracted with high K^+ PSS (40 mM) to cause depolarization, ACh still induced a concentration-dependent relaxation (Figure 2a). However, the concentration-dependent relaxation curve for ACh was shifted to the right when compared to the tissues contracted with histamine (pD_2 values 8.41 ± 0.10 vs 6.92 ± 0.13, $P<0.01$ and the maximal relaxation $103 \pm 1\%$ vs $84 \pm 5\%$, $P<0.05$, $n=6$ for both). These data suggest that the release of endothelium-derived vasodilators by ACh involves a membrane potential-dependent mechanism. On the other hand, this response to ACh was not affected by indomethacin (pD_2 values 6.92 ± 0.13 vs 6.94 ± 0.09 and the maximal relaxation $84 \pm 5\%$ vs $91 \pm 5\%$, $P>0.05$ for both, $n=6$). However, after treatment with L-NOARG alone or the combination of L-NOARG and indomethacin, the ACh-induced relaxation of vessels precontracted with high K^+ PSS was completely abolished (Figure 2a). The maximal relaxation to ACh was only $7 \pm 2\%$ and $5 \pm 1\%$ ($P<0.0$, $n=6$ for both), respectively.

Effects of guanylyl cyclase inhibitors on NO-mediated vasorelaxation

It has been suggested that the NO-induced vasorelaxation is due to increased cyclic GMP production via activation of soluble guanylyl cyclase. Increased cyclic GMP then activates cyclic GMP-dependent protein kinase, causing protein phosphorylation and eventually resulting in relaxation (Moncada *et al*., 1991; Zhao *et al*., 1997). To characterize the contribution of NO to endothelium-dependent vasorelaxation and its cellular mechanism of relaxation in RMCA, all vessels in this series of experiments were treated with indomethacin (10 μM) to block prostanoid production by cyclo-oxygenase. In arteries precontracted with histamine, ACh-induced relaxation was significantly inhibited by the guanylyl cyclase inhibitors, LY83583 and ODQ, with both the pD_2 value and maximal relaxation being decreased (Figure 2b and Table 1). In the endothelium-denuded RMCA precontracted with histamine, authentic NO (1 μM)-induced relaxation was also significantly inhibited by ODQ (10 μM, Figure 4b).

Role of K channels in NO-mediated vasorelaxation

To address the contribution of K^+ channels to ACh-induced relaxation of RMCA via release of native endothelium-derived NO, all vessels in this series of experiments were pretreated with indomethacin (10 μM), and a variety of selective K^+ channel blockers were evaluated. CTX (0.1 μM), a putative blocker of K_{Ca} channels significantly inhibited the vasorelaxation response to ACh (Figure 3a). Since CTX can inhibit K^+ channels other than BK_{Ca} (Chandy & Gutman, 1995), the effects of ITX (a more selective blocker of BK_{Ca} channels), apamin (a selective blocker of SK_{Ca} channels) and the combination of these two were also assessed. Pretreatment with ITX (0.1 μM), apamin (1 μM) or the combination of both compounds did not alter basal tone of the RMCA, but significantly inhibited the vasorelaxation response to ACh, with both the pD_2 values and maximal relaxations being decreased (Figure 3b and Table 1). After ACh-induced relaxation had reached a plateau, addition of either ITX or apamin reversed the relaxation (Figure 4a). However, this

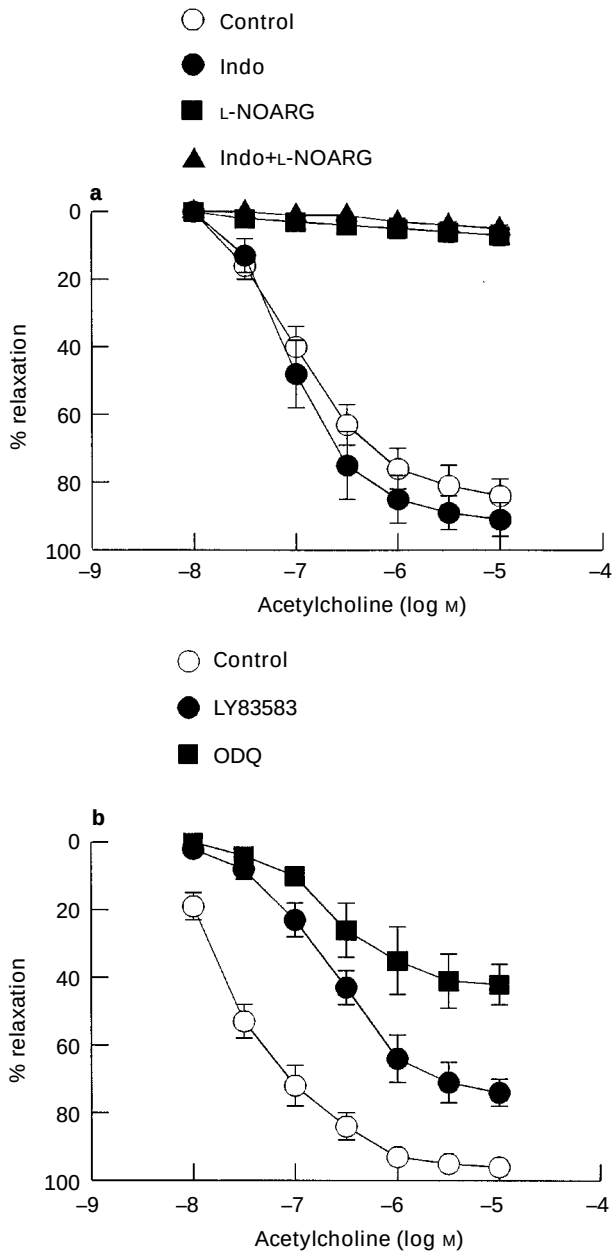

Figure 2 Mean concentration-response curves for acetylcholine in the endothelium-intact rabbit middle cerebral artery. (a) Vessels precontracted with high K^+ PSS (40 mM) in the absence and the presence of indomethacin (Indo, 10 μM), N^G-nitro-L-arginine (L-NOARG, 100 μM) or indomethacin plus N^G-nitro-L-arginine. (b) Vessels were pretreated with indomethacin (10 μM) in the normal PSS for 30 min and then precontracted with histamine (3 μM) in the absence and the presence of LY83583 (10 μM) or ODQ (10 μM). Points are the mean from 5–6 separate experiments; vertical lines show s.e.mean.

native endothelium-derived NO-induced vasorelaxation was unaffected by DTX (0.1 μM) and 4-AP (1 mM), both selective blockers of K_V channels, and glibenclamide (10 μM), a selective blocker of K_{ATP} channels, regardless of their additions before or after ACh (Figures 3c and d and 4a). Subsequent incubation with L-NOARG abolished the ACh-induced vasorelaxation, indicating the involvement of a NO-mediated pathway (Figure 3c and d and Table 1). In the endothelium-denuded RMCA precontracted with histamine, authentic NO(1 μM)-induced relaxation was also significantly inhibited by ITX and apamin, but unaffected by DTX, 4-AP and glibenclamide (Figure 4b).

Effects of adenylyl cyclase inhibitors on prostanoid-mediated vasorelaxation

It has been suggested that prostanoid-induced vasorelaxation is due to increased cyclic AMP production via activation of soluble adenylyl cyclase. Increased cyclic AMP then activates cyclic AMP-dependent protein kinase A (PKA), causing protein phosphorylation and eventually resulting in relaxation (Schubert *et al*., 1996; 1997). To characterize the contribution of prostanoid to endothelium-dependent vasorelaxation and

Table 1 Comparison of pD_2 values and maximum relaxation to acetylcholine in histamine-precontracted rabbit middle cerebral artery in the presence of indomethacin (10 μM)

	pD_2		Maximum relaxation (%)	
Treatment	*Before*	*After*	*Before*	*After*
LY83583 (10 μM)	7.44±0.09	6.76±0.10**	94±2	74±4**
ODQ (10 μM)	7.43±0.09	6.70±0.10**	94±3	42±6**
Charybdotoxin (0.1 μM)	7.44±0.15	6.94±0.09*	96±2	86±6
Iberiotoxin (0.1 μM)	7.36±0.13	6.79±0.08**	98±1	63±7**
Apamin (1 μM)	7.40±0.19	6.93±0.11*	96±2	50±5**
Iberiotoxin + apamin	7.36±0.13	5.97±0.26**	98±1	40±6**
4-Aminopyridine (1 mM)	7.43±0.13	7.40±0.13	94±3	96±4
Dendrotoxin (0.1 μM)	7.42±0.19	7.36±0.14	94±2	92±3
Glibenclamide (10 μM)	7.40±0.11	7.32±0.11	93±1	96±1
L-NOARG (100 μM)	7.40±0.11	ND	93±1	15±5**

Values are means±s.e.mean; $n=5-7$ observations. ND: the pD_2 values could not be determined due to abolition of the response. *$P<0.05$, **$P<0.01$ vs before drug treatments by Student's paired t test.

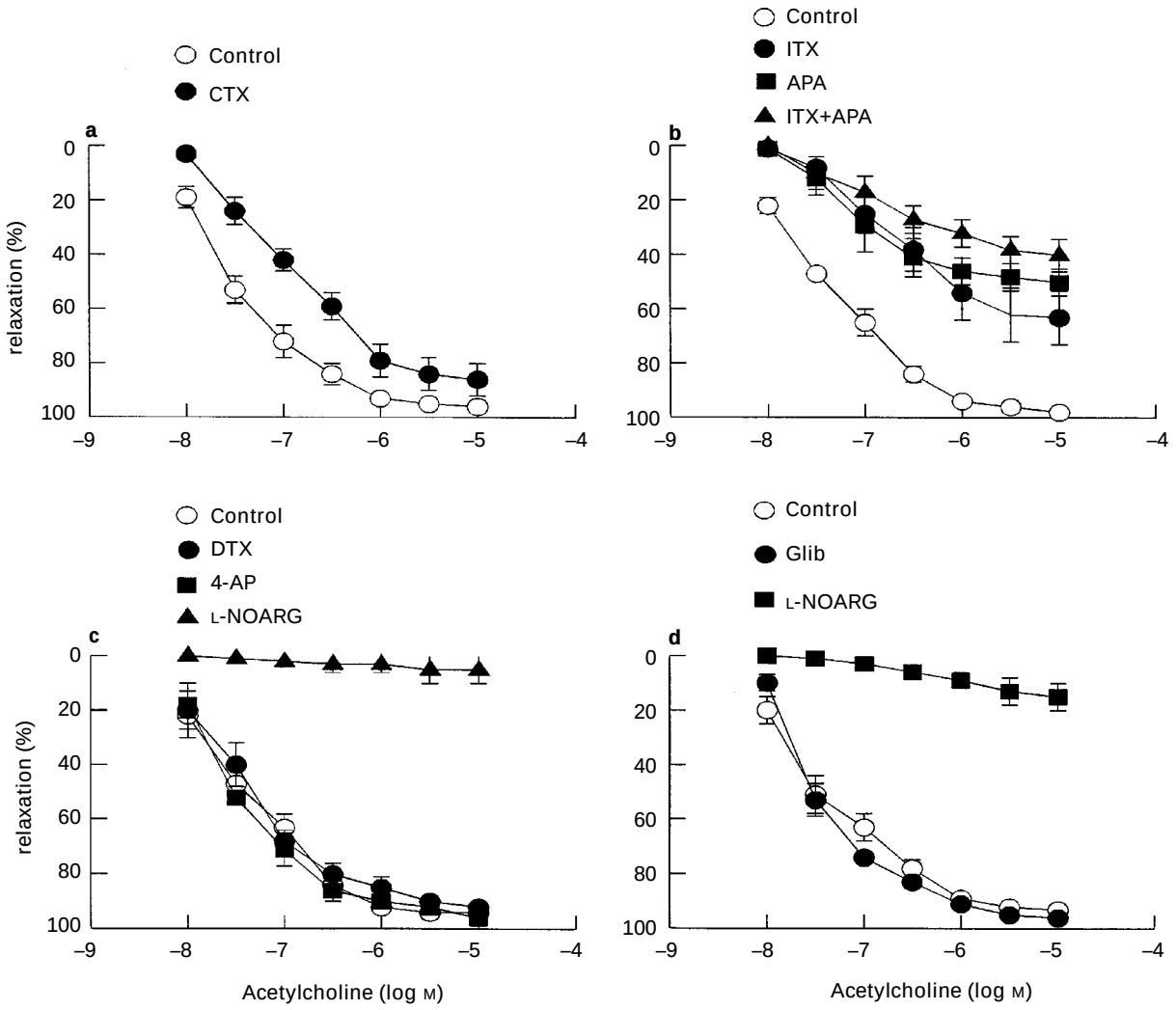

Figure 3 Mean concentration-response curves for acetylcholine in the endothelium-intact rabbit middle cerebral artery precontracted with histamine (1–3 μM) in the presence of indomethacin (10 μM) under control conditions, or in the presence of: (a) charybdotoxin (CTX, 0.1 μM); (b) iberiotoxin (ITX, 0.1 μM), apamin (APA, 1 μM) and a combination of iberiotoxin and apamin; (c) dendrotoxin (DTX, 0.1 μM), 4-aminopyridine (4-AP, 1 mM), N^G-nitro-L-arginine (L-NOARG, 100 μM); (d) glibenclamide (Glib, 10 μM), N^G-nitro-L-arginine (100 μM). Points are the mean from 5–7 separate experiments; vertical lines show s.e.mean.

its cellular mechanism of relaxation in RMCA, all vessels in this series of experiments were treated with L-NOARG (100 μM) to block NO production by NO synthase. ACh-induced vasorelaxation was significantly inhibited following inhibition of adenylyl cyclase with SQ-22536 (10 μM) or 2′,3′-DDA (30 μM) (Figure 5a and Table 2). However, ACh-induced vasorelaxation in the presence of L-NOARG was unaffected following inhibition of cytochrome P450 monooxygenase with clotrimazole (1 μM). Subsequent incubation with indomethacin abolished the ACh-induced vasorelaxation, indicating the involvement of a prostanoid-mediated pathway (Figure 5b and Table 2). In the endothelium-denuded RMCA precontracted with histamine, exogenous PGI_2 (5 μM)-induced relaxation was also significantly inhibited by 2′,3′-DDA (Figure 4c).

Role of K channels in prostanoid-mediated vasorelaxation

The contribution of K^+ channels to ACh-induced relaxation of RMCA, via release of native endothelium-derived prostanoids was assessed in RMCA pretreated with L-NOARG (100 μM). ACh-induced vasorelaxation in the presence of L-NOARG was significantly inhibited following inhibition of K_{Ca} channels with CTX (0.1 μM), ITX (0.1 μM), apamin (1 μM) or the combination of ITX and apamin (Figure 6a and b and Table 2). This native endothelium-derived prostanoid-induced vasorelaxation was also significantly inhibited following putative inhibition of K_V channels with DTX (0.1 μM) and 4-AP (1 mM) (Figure 6c and Table 2). After ACh-induced relaxation had reached a plateau, addition of ITX, apamin or DTX reversed the relaxation (data not shown). However, ACh-induced relaxation in the presence of L-NOARG was unaffected by glibenclamide (10 μM), regardless of its addition before or after ACh, but almost abolished by subsequent incubation with indomethacin, again confirming the involvement of prostanoids (Figure 6d and Table 2). In the endothelium-denuded RMCA precontracted with histamine, exogenous PGI_2 (5 μM)-induced relaxation was also significantly inhibited by ITX, apamin, DTX and 4-AP, but unaffected by glibenclamide (Figure 4c).

In endothelium-denuded RMCA precontracted with histamine, the adenylyl cyclase activator, forskolin (0.01 – 0.3 μM) induced relaxation in a dose-dependent manner (Figure 7a). It induced 86 ± 4% vasorelaxation at a concentration of 0.03 μM ($n = 6$). However, the same concentration of forskolin did not affect the tone of RMCA precontracted with high K^+ PSS. In the presence of CTX (0.1 μM) or 4-AP (1 mM), forskolin (0.03 μM) induced only 15 ± 5% or 29 ± 6% ($P < 0.01$, $n = 4$ for both) relaxation of endothelium-denuded RMCA precontracted with histamine. Forskolin-mediated vasorelaxation in endothelium-denuded RMCA was significantly inhibited by high K^+ PSS, CTX and 4-AP, again suggesting the

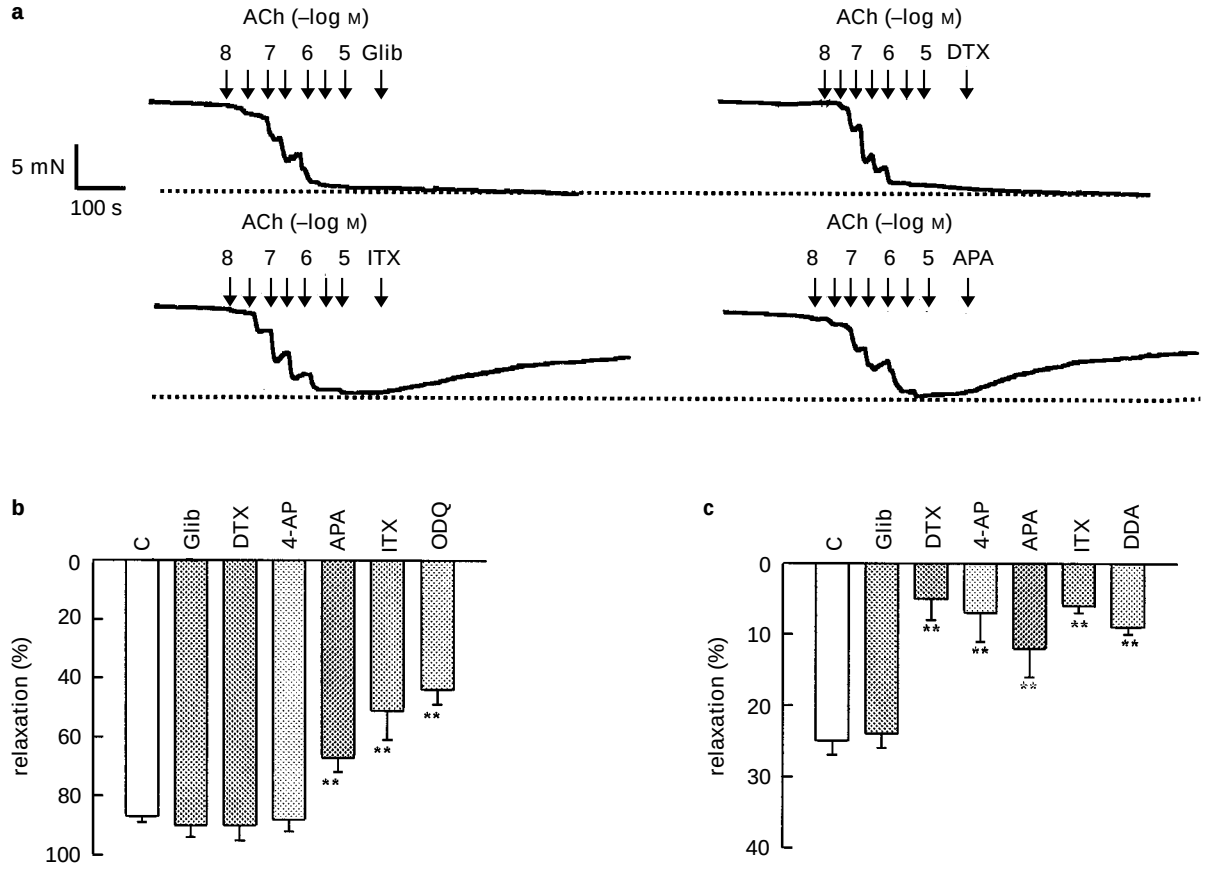

Figure 4 (a) Representative recordings showing reversible effects of K^+ channel blockers on acetylcholine (ACh)-induced relaxation of the endothelium-intact rabbit middle cerebral artery precontracted with histamine (3 μM) in the presence of indomethacin (10 μM). Glibenclamide (Glib, 10 μM), dendrotoxin (DTX, 0.1 μM), iberiotoxin (ITX, 0.1 μM) or apamin (APA, 1 μM) were added while ACh induced-relaxant responses plateaued. Acetylcholine was added in half-log concentration increments at the arrows, although the numerical values of the half-log concentrations are omitted for clarity. Effects of glibenclamide (Glib, 10 μM), dendrotoxin (DTX, 0.1 μM), 4-aminopyridine (4-AP, 1 mM), apamin (APA, 1 μM) ODQ (10 μM) and 2′,3′-DDA (DDA, 30 μM) on authentic NO (1 μM, b)- or PGI_2 (5 μM, c)-induced relaxation of the endothelium-denuded rabbit middle cerebral artery precontracted with histamine. Columns are the mean ± s.e.mean from 5 separate experiments. $^{**}P < 0.01$ vs control (C).

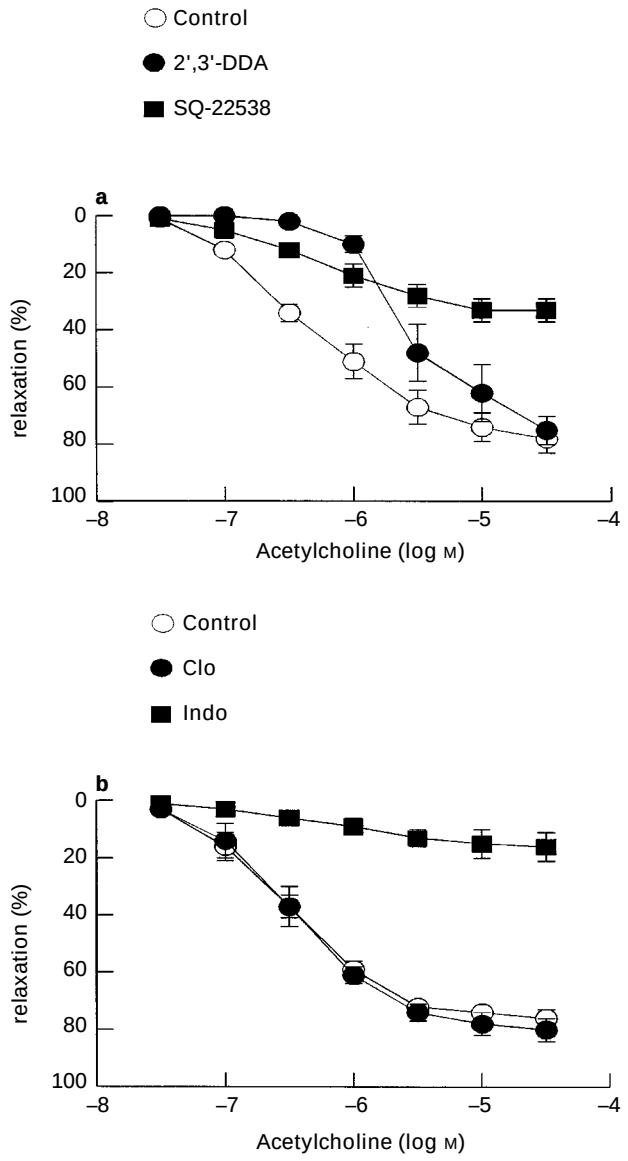

Figure 5 Mean concentration-response curves for acetylcholine in the endothelium-intact rabbit middle cerebral artery precontracted with histamine (1 μM) in the presence of N^G-nitro-L-arginine (100 μM) under control conditions, or in the presence of: (a) 2′,3′-DDA (30 μM), SQ 22,538 (10 μM); (b) clotrimazole (Clo, 1 μM), indomethacin (Indo, 10 μM). Points are the mean from 6 separate experiments; vertical lines show s.e.mean.

contribution of K^+ channels to the adenylyl cyclase-cyclic AMP pathway in RMCA (Figure 7a).

Glibenclamide did not inhibit ACh-induced endothelium-dependent vasorelaxation of RMCA in the presence of either L-NOARG or indomethacin. To provide evidence for the efficacy of glibenclamide, the effect of an activator of K_{ATP} channels, pinacidil, was studied for its ability to induce relaxation of endothelium-denuded RMCA precontracted with histamine. Glibenclamide (10 μM) significantly inhibited pinacidil-induced relaxation (Figure 7b), suggesting that it is an effective K_{ATP} channel blocker in RMCA and that K_{ATP} channels were present in the RMCA preparations that we studied.

Discussion

The data from the present study indicate that: NO and a prostanoid (possibly PGI_2) but not a non-NO/PGI_2 factor (presumably EDHF) contribute to endothelium-dependent vasorelaxation to ACh in RMCA. Furthermore, K_{Ca} channels are involved in NO-mediated relaxation of RMCA but both K_{Ca} and K_V channels are involved in PGI_2-mediated relaxation.

NO and a prostanoid but not EDHF contributes to endothelium-dependent vasorelaxation in RMCA

Three different substances, NO, PGI_2 and EDHF, have been demonstrated to contribute to endothelium-dependent vasorelaxation in response to different vasodilator agonists in different vascular beds (Gryglewski *et al*., 1986; Nagao & Vanhoutte, 1992; Parkington *et al*., 1993; Plane *et al*., 1995). The results of this study indicate that the contributions of NO and a prostanoid to endothelium-dependent vasorelaxation are important in RMCA, but the contribution of a chemically distinct EDHF is negligible. This conclusion is supported by three lines of evidence: firstly, vasorelaxation to ACh was reduced by indomethacin or L-NOARG and abolished by the combined application of indomethacin and L-NOARG in RMCA. If a distinct EDHF was involved, then an L-NOARG/indomethacin insensitive relaxation during ACh treatment should have been observed, because it is generally considered that the L-NOARG/indomethacin-insensitive component is mediated by endothelium-dependent hyperpolarization due to an EDHF (Nagao & Vanhoutte, 1992; Adeagbo & Triggle, 1993; Dong *et al*., 1997). Secondly, ACh-induced vasorelaxa-

Table2 Comparison of pD_2 values and maximum relaxation to acetylcholine in histamine-precontracted rabbit middle cerebral artery in the presence of L-NOARG (100 μM)

	pD_2		Maximum relaxation (%)	
Treatment	*Before*	*After*	*Before*	*After*
SQ 22,536 (10 μM)	6.33±0.06	6.32±0.12	74±5	33±4**
2′,3′-DDA (30 μM)	6.35±0.11	5.25±0.37**	78±6	75±5
Charybdotoxin (0.1 μM)	6.47±0.15	6.16±0.18*	82±3	37±10**
Iberiotoxin (0.1 μM)	6.42±0.08	5.83±0.21**	87±1	47±7**
Apamin (1 μM)	6.40±0.19	5.67±0.24**	85±2	33±1**
Iberiotoxin + apamin	6.42±0.08	5.53±0.23**	87±1	20±2**
4-Aminopyridine (1 mM)	6.45±0.10	5.51±0.06**	87±3	84±3
Dendrotoxin (0.1 μM)	6.50±0.10	6.03±0.04*	86±2	82±2
Clotrimazole (1 μM)	6.47±0.09	6.44±0.09	76±3	80±4
Glibenclamide (10 μM)	6.34±0.08	6.23±0.09	79±8	81±4
Indomethacin (10 μM)	6.41±0.09	ND	78±6	14±5**

Values are means ±s.e.mean; $n=6-7$ observations. ND: the pD_2 values could not be determined due to abolition of the response. *$P<0.05$, **$P<0.01$ vs before drug treatments by Student's paired t test.

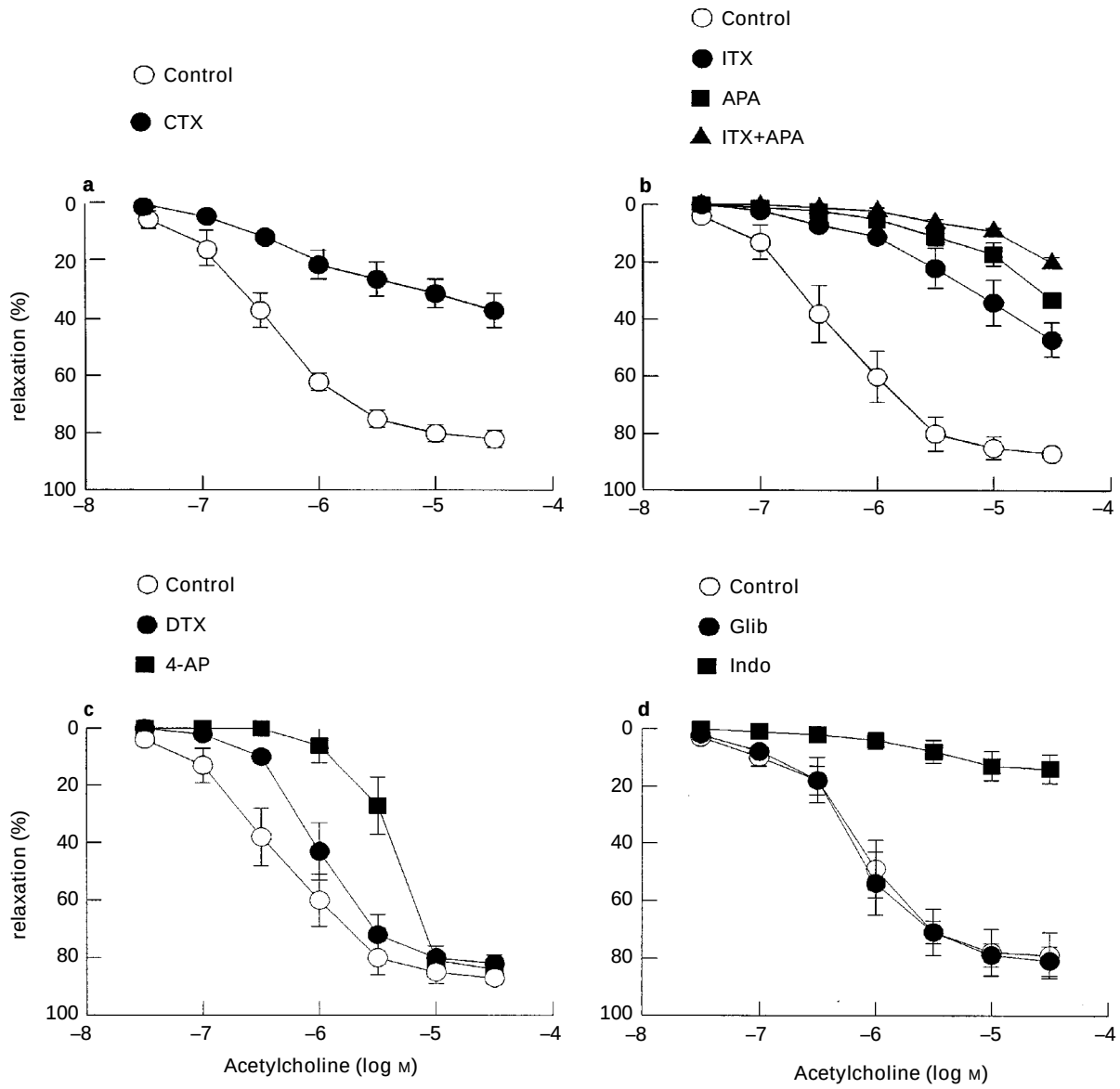

Figure 6 Mean concentration-response curves for acetylcholine in the endothelium-intact rabbit middle cerebral artery precontracted with histamine in the presence of N^G-nitro-L-arginine (100 μM) under control conditions, or in the presence of: (a) charybdotoxin (CTX, 0.1 μM); (b) iberiotoxin (ITX, 0.1 μM), apamin (APA, 1 μM), combination of iberiotoxin and apamin; (c) dendrotoxin (DTX, 0.1 μM), 4-aminopyridine (4-AP, 1 mM); (d) glibenclamide (Glib, 10 μM), indomethacin (Indo, 10 μM). Points are the mean from 6–7 separate experiments; vertical lines show s.e.mean.

tion was sensitive to either LY 83583 and ODQ, selective guanylyl cyclase inhibitors in the presence of indomethacin, or sensitive to SQ-22536 and 2,3-DDA, selective adenylyl cyclase inhibitors in the presence of L-NOARG. Thirdly, the molecular identity of EDHF is not known for certain, but recent studies provide evidence that EDHF may be a cytochrome P450 metabolite (Campbell *et al*., 1996; Chen & Cheung, 1996; Popp *et al*., 1996). Clotrimazole, an imidazole that binds to the haeme moiety of the cytochrome P450 monooxygenase to inhibit specifically the enzyme (Oyekan *et al*., 1994; Harder *et al*., 1995), did not affect the ACh-induced endothelium-dependent relaxation in RMCA. These data suggest that a cytochrome P450 metabolite (a candidate for EDHF) may not be involved in endothelium-dependent vasorelaxation to ACh in RMCA.

As we have already mentioned, there is conflicting evidence concerning the involvement of EDHF in the endothelium-dependent vasorelaxation of RMCA. Brayden (1990) suggested that hyperpolarization in RMCA during ACh treatment resulted from the release of EDHF, in which activation of K_{ATP} channels was involved. However, the sensitivity of the hyperpolarization and relaxation to NO synthase or cyclooxygenase inhibitors was not tested. Thus Brayden could not rule out the possibility that ACh-induced hyperpolarization and relaxation were mediated by NO or prostanoids (such as PGI_2). In the present study endothelium-dependent vasorelaxation to ACh in RMCA was abolished after the tissues were pretreated with a combination of L-NOARG and indomethacin. The lack of involvement of both EDHF and glibenclamide on ACh-induced vasorelaxation of RMCA in our study contrasts with the observations of Brayden (1990), but is consistent with those of Parsons *et al*. (1991) and Wellman & Bevan (1995). We cannot attribute our inability to identify an EDHF-mediated response in RMCA to the lack of specificity of the agents or conditions employed, since we were able to show that EDHF was involved in the middle cerebral

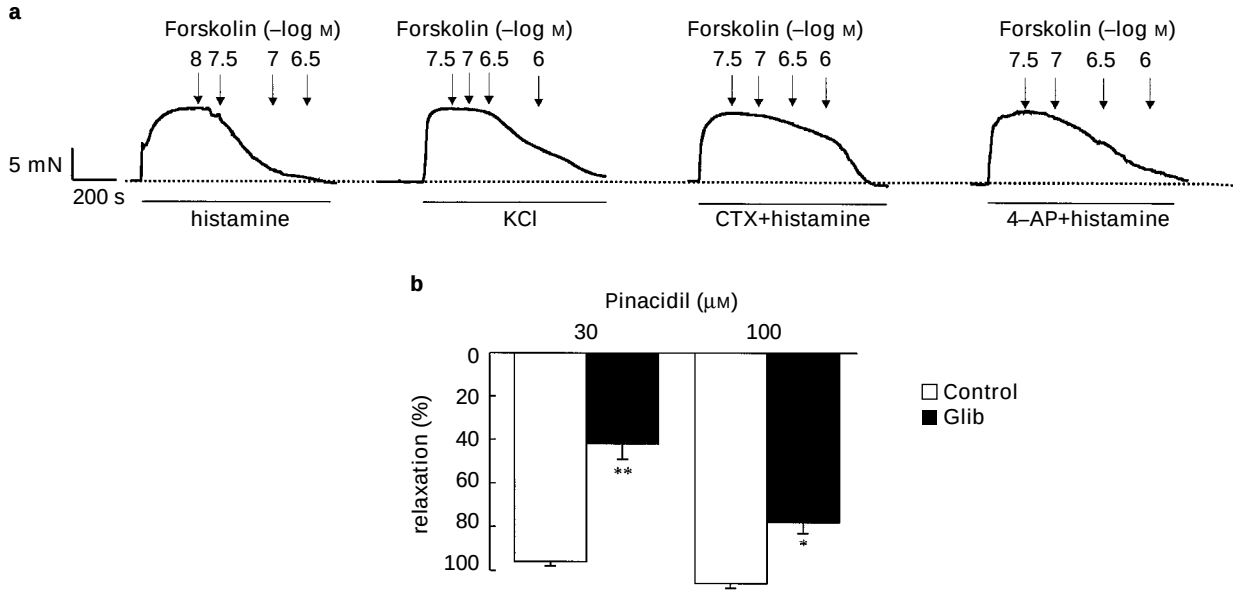

Figure 7 (a) Representative recordings of forskolin-induced relaxation of the endothelium-denuded rabbit middle cerebral artery precontracted with histamine (1–3 μM) or high K^+ PSS (40 mM) in the absence and the presence of charybdotoxin (CTX, 0.1 μM) or 4-aminopyridine (4-AP, 1 mM). (b) Inhibitory effect of glibenclamide on pinacidil-induced relaxation of the endothelium-denuded rabbit middle cerebral artery precontracted with histamine. Columns which are the mean ± s.e.mean from 5 separate experiments, represent the effects under control conditions (control) and in the presence of glibenclamide (Glib, 10 μM). $*P<0.05$, $**P<0.01$ vs control.

artery of guinea-pigs (Dong *et al*., 1996) and rabbit carotid artery (Dong *et al*., 1997) under similar experimental conditions. Rather, we attribute this difference to species variations in the contribution of NO, PGI_2 and EDHF to endothelium-dependent vasodilatation. In the present study, we did not observe any effect of glibenclamide on ACh-induced vasorelaxation in RMCA. In order to provide evidence for the efficacy of glibenclamide, an activator of K_{ATP} channels, pinacidil, was used in the present study to induce hyperpolarization and hence vasorelaxation. Glibenclamide (10 μM), did not have any effect on ACh-induced vasorelaxation of endothelium-intact RMCA, but significantly inhibited pinacidil-induced vasorelaxation of endothelium-denuded RMCA. In a recent study, Yamakawa *et al*., (1997) showed the presence of an indomethacin- and nitric oxide synthase inhibitor-resistant hyperpolarizing factor that was responsible for ACh-induced relaxation of the distal rabbit middle cerebral artery. This EDHF was sensitive to apamin. Differences between experimental conditions could explain these findings which differ from the present study e.g. proximal vs distal middle cerebral artery, ring vs strip set up, different level of basal and/or induced tone or depolarization, or differences in the strain of rabbit used (Japanese White vs New Zealand White). Indomethacin was present throughout all the vasorelaxant protocols so the contribution of PGI_2 to ACh-induced relaxation could not be assessed.

K_{Ca} channels are involved in NO-mediated vasorelaxation of RMCA

It has been established that NO activates guanylyl cyclase, and, hence, elevates the intracellular cyclic GMP level in many kinds of tissues. Thus, we employed inhibitors of guanylyl cyclase to characterize the second messenger pathway activated by the NO in cerebral artery. LY83583 inhibits soluble guanylyl cyclase via the generation of hydroxyl radicals (Kontos & Wei, 1993) and superoxide anions (Luond *et al*., 1993). These free radicals will also inactivate NO (Barbier & Lefebvre, 1992). LY83583 has also been shown to inhibit nitric oxide synthase (Mulsch *et al*., 1988). However, ODQ directly inhibits soluble guanylyl cyclase (Schrammel *et al*., 1996), though not particulate guanylyl cyclase or adenylyl cyclase (Garthwaite *et al*., 1995). Also, it does not inhibit nitric oxide synthase, generate superoxide anions or affect the autooxidation of NO (Garthwaite *et al*., 1995). Thus we have used drugs that utilize two distinct mechanisms to inactivate guanylyl cyclase. In the presence of indomethacin, to abolish the contribution of prostanoids by inhibiting both cyclo-oxygenase and their binding to PGI_2 receptors on target cell membranes (Parfenova *et al*., 1995; Leffler, 1997), ACh-induced relaxation was inhibited by LY83583. We also studied the effects of ODQ and found that ACh-induced relaxation in the presence of indomethacin was significantly inhibited by ODQ. In addition, histamine precontracted endothelium-denuded RMCA relaxed to authentic NO and this relaxation was significantly inhibited by ODQ. Our data indicate that ACh-induced vasorelaxation of RMCA in the presence of indomethacin is via release of native endothelium-derived NO and the subsequent activation of guanylyl cyclase.

The cellular mechanism underlying the NO-mediated relaxation response of vascular smooth muscle is only partially understood, especially with respect to the subtype of K^+ channel(s) involved. NO mediates the activation of both K_{Ca} and K_V channels in the bovine coronary arterial smooth muscle and the rat pulmonary artery (Li *et al*., 1997; Zhao *et al*., 1997), but it activates either K_{Ca} in the guinea-pig pulmonary artery (Bialecki & Stinson-Fisher, 1995) and the rabbit carotid artery (Najibi & Cohen, 1995) or K_{ATP} channels in the rabbit mesenteric artery (Murphy & Brayden, 1995). These data suggest that the subtypes of K^+ channels involved in NO-mediated relaxation of peripheral arteries appear to be both tissue- and species-dependent and the same conclusion can be reached for cerebral vessels. However, subtypes of K^+ channels involved

in NO-mediated relaxation of cerebral resistance vessels are not fully understood. Our data indicate that changes in membrane potential may contribute to the effect of NO in middle cerebral arteries. Glibenclamide, a blocker of K_{ATP} channels, administered either before or after ACh, was without any effect on ACh-induced relaxation of RMCA in the presence of indomethacin. However, CTX, a putative blocker of K_{Ca} channels (Haylett & Jenkinson, 1990; Nelson & Quayle, 1995), inhibited ACh-induced relaxation, suggesting that a membrane hyperpolarization due to K_{Ca} channel activation is involved. Since CTX is also known to affect K_V channels (Chandy & Gutman, 1995), we also determined the effects of iberiotoxin and apamin, which are more specific blockers of BK_{Ca} or SK_{Ca} channels (Galvez *et al*., 1990; Haylett & Jenkinson, 1990), and both produced a significant inhibition of ACh-induced relaxation. A combination of iberiotoxin and apamin also significantly inhibited NO-mediated relaxation. In contrast, 4-AP and DTX, more specific blockers of K_V channels (Corrette *et al*., 1991; Nelson & Quayle, 1995), were without any inhibitory effect. These data clearly indicate that K_{Ca} channels (both BK_{Ca} and SK_{Ca}) rather than K_{ATP} and K_V channels are involved in NO-mediated relaxation of RMCA. In the endothelium-denuded RMCA, authentic NO-induced relaxation was also significantly inhibited by ITX and apamin, but unaffected by glibenclamide, DTX and 4-AP, again indicating that BK_{Ca} and SK_{Ca} are of importance for the NO-mediated response in cerebral arteries.

There are two possible explanations for the effects of K_{Ca} channel blockers: firstly, these toxins could inhibit NO synthesis/release by blocking K_{Ca} channels on endothelial cells. Thus, they would prevent ACh-mediated endothelial cell hyperpolarization and prevent the increased Ca^{2+} entry through non-selective cation channels by reducing the electrochemical driving force for Ca^{2+} influx. Alternatively, K_{Ca} channel blockers may block the action of NO at the level of the smooth muscle cells, inhibition of K_{Ca} channels would prevent hyperpolarization and closure of voltage-dependent L-type Ca^{2+} channels. However, an effect on endothelial cell membrane potential seems unlikely, since an L-NOARG-sensitive relaxation was observed in the RMCA in the presence of high K^+ PSS (Figure 2a). NO is known to activate smooth muscle BK_{Ca} channels directly by altering the redox state of the channel protein or an associated regulatory subunit (Bolotina *et al*., 1994), or indirectly, via a phosphotransferase reaction involving cyclic GMP-dependent protein kinase (Robertson *et al*., 1993). Also in the endothelium-denuded RMCA, authentic NO-induced relaxation was significantly inhibited by ITX and apamin, again indicating that K_{Ca} channel blockers block the action of NO at the level of the smooth muscle cells.

Both K_{Ca} and K_V channels are involved in PGI_2-mediated vasorelaxation of RMCA

In the presence of L-NOARG to abolish the contribution of endogenously synthesized NO, ACh induced an indomethacin-sensitive relaxation in RMCA, which was significantly reduced by the adenylyl cyclase inhibitors, SQ 22,536 and 2′, 3′-DDA (Tamaoki *et al*., 1993; Koh *et al*., 1995), suggesting that a prostanoid released from endothelial cells contributes to endothelium-dependent relaxation of the RMCA, via an adenylyl cyclase-cyclic AMP pathway. SQ22536 and 2′, 3′ DDA are thought to be specific inhibitors of adenylyl cyclase by interaction with the 'P'-region of the cyclase. SQ22536 does not inhibit phosphodiesterases as does another adenylyl cyclase inhibitor, MDL 12330A (Lippe & Ardizonne, 1991) and has been used in concentrations as high at 100 μM (McLean & Coupar, 1996). In addition, the fact that forskolin-induced relaxation was inhibited by CTX and 4-AP in a similar manner to that of endogenous prostanoids argues for the involvement of adenylyl cyclase in this pathway. We cannot be certain that in our studies the indomethacin-sensitive product is PGI_2. However, given the extensive literature demonstrating endothelial cell synthesis and release of PGI_2 (Gryglewski *et al*., 1986; Parfenova *et al*., 1995) and given that PGI_2 is a potent vasodilator acting via the cyclic AMP pathway (Shaul *et al*., 1992), we believe that it is likely that the prostanoid is PGI_2.

The cellular mechanism underlying the PGI_2-mediated relaxation of vascular smooth muscle is only partially understood. Siegel *et al*. (1989) and Parkington *et al*. (1993, 1995) showed that PGI_2 and iloprost (a PGI_2 analogue), caused hyperpolarization in canine carotid artery and guinea-pig coronary artery and concluded that PGI_2 could be classified as a K^+ channel opener. However, the subtypes of K^+ channels involved in PGI_2-mediated relaxation of peripheral vessels appear to depend on tissue source and species. PGI_2 activates both K_{Ca} and K_{ATP} channels in rat tail artery (Schubert *et al*., 1997), but it activates either BK_{Ca} channels (Siegel *et al*., 1989; Minami *et al*., 1993) in canine carotid artery and porcine coronary artery or 4-AP-sensitive delayed rectifier K^+ channels in bovine coronary artery (Li *et al*., 1997). Although PGI_2 is known to be an important modulator of the cerebral circulation (Parfenova *et al*., 1995; Leffler, 1997), there is little known about the subtypes of K^+ channels involved in PGI_2-mediated relaxation of cerebral resistance vessels. In the present study, glibenclamide had no significant effect on ACh-induced relaxation of the RMCA in the presence of L-NOARG. However, CTX inhibited the ACh-induced relaxation, suggesting that K_{Ca} and K_V channels may be involved in the PGI_2-mediated relaxation in RMCA. In support of this hypothesis, iberiotoxin and apamin, more specific blockers of BK_{Ca} or SK_{Ca} channels, respectively, both produced significant reduction of ACh-induced relaxation. A combination of iberiotoxin and apamin also significantly inhibited PGI_2-mediated relaxation. Similarly, 4-AP and DTX, more specific blockers of K_V channels, also significantly inhibited ACh-induced relaxation. These data clearly indicate that both K_{Ca} channels (both BK_{Ca} and SK_{Ca}) and K_V channels rather than K_{ATP} are involved in PGI_2-mediated relaxation of RMCA. In the endothelium-denuded RMCA, exogenous PGI_2-induced relaxation was also significantly inhibited by ITX, apamin, 4-AP and DTX, but unaffected by glibenclamide, again indicating that K_{Ca} and K_V channels are of importance for the PGI_2-mediated response in cerebral arteries. It has been suggested that cyclic AMP-dependent protein kinase A (PKA) is the mediator of PGI_2-induced relaxation in peripheral and cerebral arteries (Taguchi *et al*., 1995; Schubert *et al*., 1996; 1997; Leffler, 1997). Our data showing that adenylyl cyclase inhibitors significantly inhibit the vasorelaxation of RMCA induced by PGI_2, either released from endothelium or added exogenously, and that the adenylyl cyclase activator, forskolin-induced vasorelaxation was also significantly inhibited by high K^+ PSS, CTX and 4-AP, suggest that PGI_2 may activate K^+ channels in the RMCA via a cyclic AMP-dependent protein kinase-mediated phosphorylation (Schubert *et al*., 1996; 1997).

In conclusion, this study indicates that: (a) NO and a prostanoid (likely PGI_2) are the two key EDRFs released by ACh in the RMCA, and that (b) their cellular effects are mediated by guanylyl and adenylyl cyclases with the targets, in part, being K_{Ca} and K_{Ca} and K_V channels, respectively.

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