Adrenomedullin-Induced Endothelium-Dependent Relaxation in Porcine Ciliary Arteries

Eike S. Dettmann, Ineta Vysniauskiene, Renyi Wu, Josef Flammer, and Ivan O. Haefliger

Purpose. To investigate adrenomedullin-induced relaxation in isolated porcine ciliary arteries.

Methods. In a myograph system (isometric force measurement), precontracted vessels (~0.1 μM U46619; thromboxane A_2 analogue or ~10 nM endothelin-1) were exposed, in a cumulative manner, to increasing concentrations of adrenomedullin (1 nM to 1 μM) in the presence or absence of different drugs. Some experiments were conducted in vessels with nonfunctional (intentionally mechanically damaged) endothelium.

Results. Adrenomedullin evoked marked relaxation [maximum relaxation (Rel_{max}): 85.5 ± 3.0%; negative log M concentration inducing 50% of Rel_{max} (pD_2): 7.4 ± 0.1] in comparison to time-controls (Rel_{max}: 19.2 ±4.8%; $P < 0.001$). Relaxation was inhibited by 3 μM CGRP[8-37] ($CGRP_1$ receptor antagonist; Rel_{max}: 27.2% ± 5.3%; $P < 0.001$) but not by 3 μM adrenomedullin[22-52] (presumed adrenomedullin receptor antagonist; $P = 0.75$). Adrenomedullin-induced relaxation was less pronounced in nonfunctional endothelium vessels (Rel_{max}: 67.6% ± 3.1%; pD_2: 6.9 ± 0.1; $P < 0.01$). In vessels with functional endothelium, relaxation was not significantly influenced by 0.1 mM N^G-nitro-L-arginine methyl ester (L-NAME; a nitric oxide synthesis inhibitor), 10 μM indomethacin (a cyclooxygenase inhibitor), or 10 μM 17-octadecynoic acid (a cytochrome P_{450} inhibitor). In contrast, relaxation was significantly inhibited by either 10 mM tetraethylammonium (nonselective potassium channel inhibitor; $P < 0.01$) or 50 nM apamin (small conductance potassium channel inhibitor), together with 50 nM charybdotoxin (large and intermediate potassium channel inhibitor; $P < 0.01$). In the presence of these potassium channel inhibitors, the amount of relaxation was not significantly different ($P > 0.50$) from that observed in vessels with nonfunctional endothelium.

Conclusions. In isolated porcine ciliary arteries, adrenomedullin induces relaxation that involves $CGRP_1$ receptors and is in part endothelium dependent. Endothelium-dependent relaxation was blocked by some potassium channel inhibitors, suggesting the possible release of an endothelium-derived hyperpolarizing factor (EDHF). (*Invest Ophthalmol Vis Sci.* 2003;44: 3961–3966) DOI:10.1167/iovs.02-1312

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Adrenomedullin is a hypotensive 52-amino-acid peptide with structural similarities to calcitonin gene-related peptide (CGRP). Originally isolated from human pheochromocytoma,[1] the peptide has also been found in several tissues, such as the adrenal medulla, the kidney, and the lung, as well as in the cerebral cortex and the spinal cord.[2,3] Under physiologic conditions considerable amounts of the peptide are present in the blood, suggesting that adrenomedullin may play a role in the control of the circulation. In patients with heart failure, hypertension, or septic shock, increased plasma levels of adrenomedullin have been measured.[4] In the eye, increased concentrations of adrenomedullin have been observed in the aqueous humor of patients with primary open-angle glaucoma.[5]

Adrenomedullin, which can be produced and secreted by various types of cells, such as cardiomyocytes, fibroblasts, macrophages, neurons, glial cells, retinal pigment epithelial cells, vascular smooth muscle cells, and vascular endothelial cells[6] has vasodilatory properties. In some vessels, this relaxation is in part mediated by the release of endothelium-derived relaxation factors.[7–9] Indeed, the vascular endothelium has the ability to release vasorelaxing substances, such as nitric oxide (NO), prostacyclin, and the putative endothelium-derived hyperpolarizing factor (EDHF). It has been reported that, in kidney arteries of the dog,[10] or the hindquarters vascular bed of the rat,[11] part of the adrenomedullin-induced relaxation is mediated by NO. In the human coronary arterioles it has further been shown that adrenomedullin elicits vasodilatation in part through the production of NO and in part through the activation of potassium channels.[12] In contrast, in some vessels, such as canine central retinal artery, an endothelium-dependent adrenomedullin-induced relaxation was not demonstrated.[13] It has been reported that part of the biological activities of adrenomedullin are mediated by CGRP receptors, but the question of whether a specific adrenomedullin receptor exists is still debated.[14,15]

The major purpose of the present study was to investigate the role that is played by endothelium-derived relaxation factors in the relaxation induced by adrenomedullin in isolated porcine ciliary arteries.

Materials and Methods

Vessel Preparation

Porcine eyes were obtained from an abattoir immediately after death. In cold modified Krebs-Ringer solution (NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 11.1 mM, and EDTA 0.026 mM), ciliary arteries were dissected and cut into 2-mm segments.[16] In an organ chamber, two 30-μm tungsten wires were passed through the vessel's lumen and attached to a force transducer for isometric force measurements (Myo-Interface; JP Trading, Aarhus, Denmark). Vessels were left in place for 30 minutes in the modified Krebs-Ringer solution (95% O_2, 5% CO_2, 37°C) before being stretched in a stepwise fashion to reach their optimal passive tension (~950 mg).[17] Vessels were then exposed several times to 100 mM KCl until they showed reproducible contractions (i.e., <10% variation between two successive KCl-induced contractions). The functional integrity of the endothelium was assessed by relaxing the vessels with bradykinin. The endothelial function was considered to be

intact if bradykinin (100 nM–1 μM) evoked 80% relaxation of a vessel precontracted with the thromboxane A_2 analogue U46619 (0.1 μM).[18] In some vessels the integrity of the endothelium was compromised by rubbing the luminal surface of the vessels with a human scalp hair. In these vessels with a nonfunctional endothelium, bradykinin evoked no relaxation. All vessels were then washed out with modified Krebs-Ringer solution before any of the experimental protocols described below were conducted.

Experimental Protocol

Arteries precontracted with U46619 (~0.1 μM) were exposed to increasing concentrations of adrenomedullin (1 nM to 1 μM). Concentration–response curves were constructed in the absence or in the presence of: (1) CGRP[8-37] (1 and 3 μM), a $CGRP_1$ receptor antagonist; (2) adrenomedullin[22-52] (AM[22-52], 1 and 3 μM), an adrenomedullin receptor antagonist; (3) N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM), an inhibitor of NO formation; (4) tetraethylammonium (TEA, 10 mM), a nonselective potassium channel inhibitor; (5) a combination of Ca^{2+}-dependent potassium channel inhibitors, apamin (50 nM), which inhibits small-conductance potassium channels, and charybdotoxin (ChTX, 50 nM), which inhibits large- and intermediate-conductance potassium channels; and (6) 17-octadecynoic acid (17-ODYA, 10 μM), a cytochrome P_{450} inhibitor. Furthermore, concentration–response curves to sodium nitroprusside (SNP, 1 nM to 0.3 mM) were constructed in the absence or presence of CGRP[8-37] (0.1 μM). In one additional set of experiments, vessels were precontracted with endothelin-1 (~10 nM) and concentration–response curves to adrenomedullin were constructed in the absence or presence of indomethacin (10 μM), an inhibitor of cyclooxygenase, because it has been reported that the latter can significantly attenuate the contractions induced by U46619.[19] The preincubation time for all these drugs was 30 minutes.

Drugs

Bradykinin, L-NAME, indomethacin, U46619, SNP, 17-ODYA, TEA, apamin, and ChTX were purchased from Sigma-Aldrich (Buchs, Switzerland). Adrenomedullin (human), adrenomedullin[22-52], and CGRP[8-37] were bought from Bachem AG (Bubendorf, Switzerland) and endothelin-1 from Novabiochem (Laufelfingen, Switzerland). All drugs were dissolved in distilled water except indomethacin, 17-ODYA, and endothelin-1, which were dissolved in 10 μM Na_2CO_3, ethanol, and Krebs solution containing 0.05% bovine serum albumin, respectively. Concentrations are expressed as final molar concentrations in the organ chambers.

Statistical Analysis

Results are given as the mean ± SEM, with n equaling the number of eyes (one eye per animal) studied. Relaxation is expressed as a percentage of the maximum contraction evoked by U46619 (~0.1 μM) or endothelin-1 (~10 nM). The EC_{50}, or the concentration causing 50% of the maximum relaxation (Rel_{max}), is expressed as a negative log M concentration (pD_2). Statistical comparisons were conducted with a one-way ANOVA multiple comparison followed by the Bonferroni test, with a two-tailed $P < 0.05$ considered to be statistically significant.

Results

Adrenomedullin-Induced Relaxation

In porcine ciliary arteries precontracted with the thromboxane analogue U46619 (~0.1 μM), adrenomedullin (1 nM to 1 μM) evoked potent concentration-dependent relaxation (Rel_{max}: 85.5% ± 3.0%, pD_2 = 7.4 ± 0.1). The adrenomedullin-induced relaxation was inhibited by the $CGRP_1$ receptor antagonist CGRP[8-37] in a dose-dependent manner (1 μM, Rel_{max}: 64.7% ± 4.8%; 3 μM Rel_{max}: 27.2% ± 5.3%, $P < 0.001$ vs. control, Fig. 1A). The relaxation evoked by adrenomedullin in the presence of 3 μM CGRP[8-37] was not significantly different ($P = 0.62$) from the spontaneous loss of tone observed in time-control experiments (Rel_{max}: 19.2% ± 4.8%, Fig. 1A). In contrast, the relaxation induced by adrenomedullin was not affected by AM[22-52], a drug that has been reported to have some adrenomedullin receptor antagonist properties[14,15] (1 μM, Rel_{max} = 112.0% ± 4.6%; 3 μM Rel_{max} = 115.7% ± 9.4%, $P = 0.75$ vs. control; Fig. 1B). To exclude an unspecific inhibitory effect of CGRP[8-37] on the relaxing properties of the vessels, the effect of CGRP[8-37] was also tested on the relaxation induced by SNP. The relaxation evoked by SNP (1 nM to 0.3 mM; $n = 10$; Rel_{max} = 89.3% ± 4.7%) was not significantly affected by CGRP[8-37] (10 μM; $n = 7$; Rel_{max} = 76.6% ± 8.8%, $P = 0.22$ vs. control, Fig. 2). These results tend to indicate that adrenomedullin relaxes porcine ciliary arteries essentially

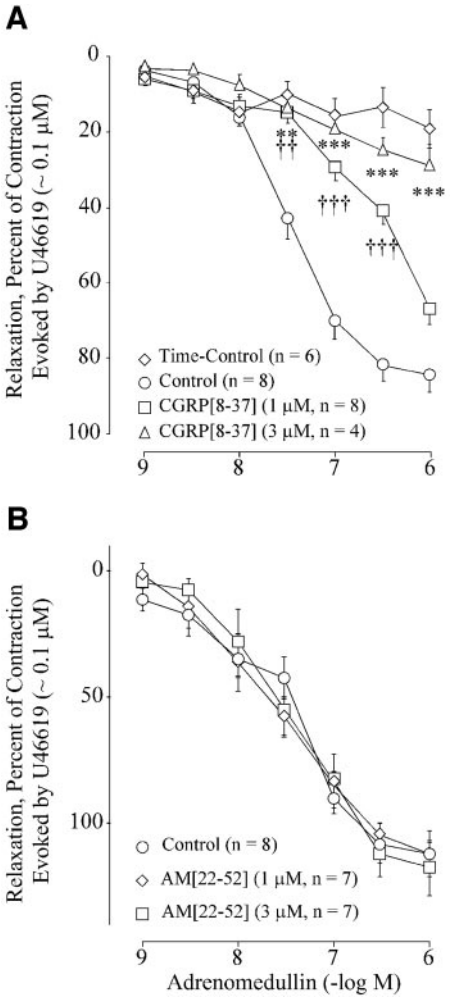

Figure 1. Effect of the $CGRP_1$ receptor antagonist CGRP[8-37] and the adrenomedullin receptor antagonist adrenomedullin[22-52] on adrenomedullin-induced relaxation in isolated porcine ciliary arteries precontracted with the thromboxane A_2 analogue U46619. (**A**) In comparison to adrenomedullin-induced relaxation without any antagonist (control), increasing concentrations of CGRP[8-37] significantly blunted the relaxation evoked by adrenomedullin. The inhibition of the adrenomedullin-induced relaxation evoked by 3 μM CGRP[8-37] was not significantly different from the spontaneous loss of tone observed after precontraction with approximately 0.1 μM U46619 (time-control). (**B**) AM[22-52] had no significant effect on adrenomedullin-induced relaxation. Control versus 3 μM CGRP[8-37], $^{**}P < 0.01$; $^{***}P < 0.001$; control versus 1 μM CGRP[8-37], $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$.

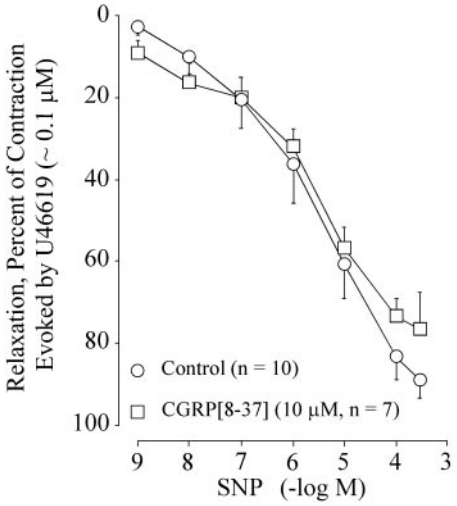

FIGURE 2. Effect of the $CGRP_1$ receptor antagonist CGRP[8-37] on SNP-induced relaxation in isolated porcine ciliary arteries precontracted with the thromboxane A_2 analogue U46619. CGRP[8-37] had no significant effect on SNP-induced relaxation.

through the activation of $CGRP_1$ receptors or receptors that have a common structural homology with $CGRP_1$ receptors.

Endothelium-Dependent Adrenomedullin-Induced Relaxation

In U46619 (~0.1 μM) precontracted vessels with a nonfunctional endothelium (intentionally and mechanically damaged), relaxation evoked by adrenomedullin (1 nM to 1 μM) was significantly blunted (Rel_{max} = 67.6% ± 3.1%, pD_2 = 6.9 ± 0.1, $P < 0.01$; Fig. 3) demonstrating that, in porcine ciliary arteries, relaxation induced by adrenomedullin was in part endothelium dependent.

L-NAME, Indomethacin, and Adrenomedullin-Induced Relaxation

In U46619 (~0.1 μM) precontracted vessels relaxation evoked by adrenomedullin was not significantly affected by the presence of the inhibitor of NO formation L-NAME (0.1 mM) at a concentration known to inhibit endothelium-dependent NO production[18] (Fig. 4A). Relaxation evoked by adrenomedullin was also unaffected by the presence of indomethacin in endothelin-1 (~10 nM) precontracted vessels at a concentration of indomethacin reported to inhibit cyclooxygenase[20] (Fig. 4B). These results demonstrate that adrenomedullin does not relax porcine ciliary arteries through the release of nitric oxide or a prostaglandin produced after cyclooxygenase activation.

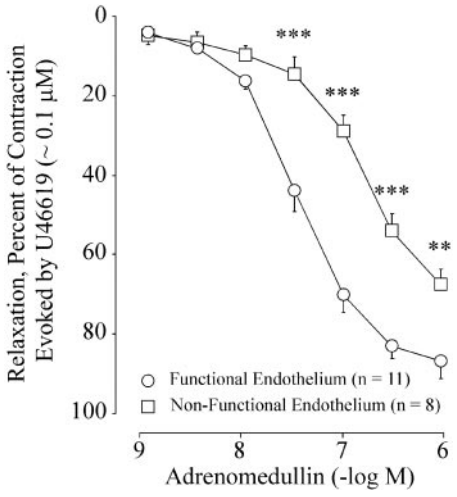

FIGURE 3. Endothelium-dependent and endothelium-independent adrenomedullin-induced relaxation in isolated porcine ciliary arteries precontracted with the thromboxane A_2 analogue U46619. The adrenomedullin-induced relaxation was partially but significantly blunted in vessels with a nonfunctional endothelium (mechanically and intentionally damaged). $^{**}P < 0.01$; $^{***}P < 0.001$.

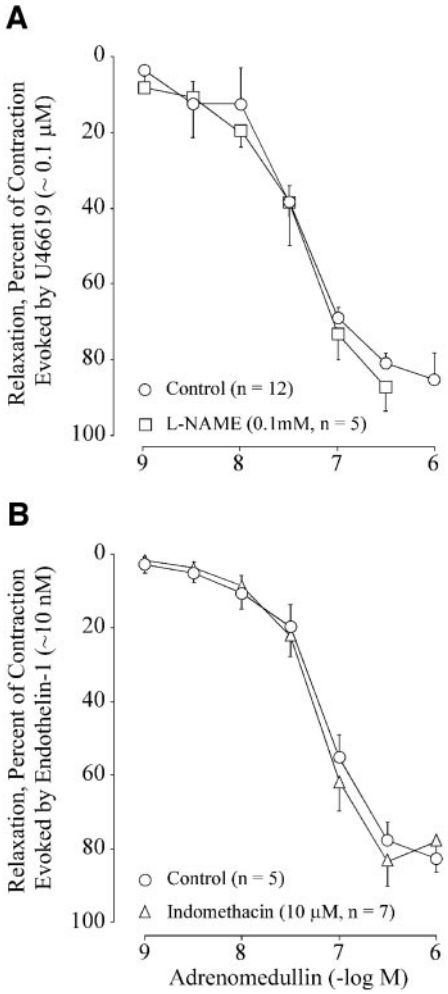

FIGURE 4. Effect on the relaxation evoked by adrenomedullin of drugs considered to inhibit the release of some endothelium-dependent relaxation factors, such as the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, **A**) or the cyclooxygenase inhibitor indomethacin (**B**). Neither L-NAME nor indomethacin had a significant effect on the relaxation induced by adrenomedullin.

EDHF and Adrenomedullin-Induced Relaxation

To determine whether in porcine ciliary arteries, relaxation induced by adrenomedullin involves the activation of potassium channels, adrenomedullin concentration–response curves were constructed, in U46619 (~0.1 μM) precontracted vessels, in the absence or in the presence of different potassium channel inhibitors. First, the effect of TEA (10 mM), a nonselective potassium channel inhibitor was investigated. Then, experiments were conducted with a combination of Ca^{2+}-dependent potassium channel inhibitors, apamin (50 nM), a small-conductance potassium channel inhibitor and ChTX (50 nM), a large- and intermediate-conductance potas-

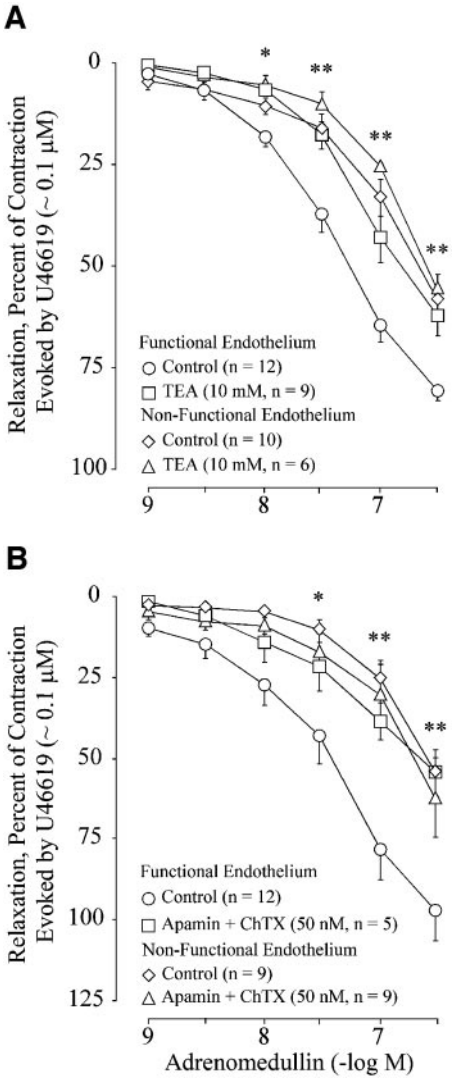

FIGURE 5. Effect of different potassium channel inhibitors on the relaxation evoked by adrenomedullin in vessels with either a functional or nonfunctional endothelium (mechanically and intentionally damaged) in isolated porcine ciliary arteries precontracted with the thromboxane A_2 analogue U46619. (**A**) TEA, a nonselective potassium channel inhibitor, significantly inhibited the relaxation evoked by adrenomedullin only in vessels with a functional endothelium, not in those with a nonfunctional endothelium. (**B**) Apamin, a small-conductance potassium channel inhibitor together with ChTX, a large and intermediate-conductance potassium channel inhibitor, significantly inhibited only the relaxation evoked by adrenomedullin in vessels with a functional endothelium but not in those with a nonfunctional endothelium. Control (functional endothelium) versus any one of the other three groups: $^{*}P < 0.05$; $^{**}P < 0.01$.

sium channel inhibitor. In vessels with a functional endothelium, relaxation evoked by adrenomedullin was significantly blunted by TEA (Rel_{max} = 62.1% ± 5.0%, $P < 0.01$; Fig. 5A), as well as by apamin together with ChTX (Rel_{max} = 53.8% ± 6.8%, $P < 0.01$; Fig. 5B). Such an inhibitory effect of TEA, or apamin+ChTX on adrenomedullin-induced relaxation was not observed in vessels with a nonfunctional endothelium. In vessels with a functional endothelium, the relaxation evoked by adrenomedullin in the presence of the potassium channel inhibitors was not significantly different ($P > 0.50$) from the relaxation observed in vessels with a nonfunctional endothelium (Fig. 5). These results suggest that the endothelium-dependent relaxation induced by adrenomedullin involves the activation of potassium channels, suggesting the possible presence of an EDHF.

It has been reported that arachidonic acid metabolites from the cytochrome P_{450} pathway can act as an EDHF and that the cytochrome P_{450} inhibitor 17-ODYA at a concentration of 3 μM was able to reduce acetylcholine-induced vasodilation in rabbit carotid artery.[21] In porcine ciliary arteries adrenomedullin-induced relaxation was not significantly affected by 10 μM 17-ODYA (Fig. 6), suggesting that a cytochrome P_{450}–derived metabolite of arachidonic acid is probably not involved in the EDHF-induced activation of potassium channels.

DISCUSSION

In the present study, by the activation of $CGRP_1$ receptors, adrenomedullin induced marked relaxation in isolated porcine ciliary arteries that was in part endothelium-dependent. The endothelium-dependent relaxation evoked by adrenomedullin was blocked by some potassium channel inhibitors suggesting the involvement of an EDHF.

The amino acid sequence of adrenomedullin has some homology with calcitonin gene-related peptide (CGRP), and adrenomedullin is therefore considered to be a member of the CGRP superfamily. In the present study the relaxing effect of adrenomedullin was blocked by the $CGRP_1$ receptor antagonist CGRP[8-37], indicating that the relaxing effect of adrenomedullin is mainly mediated by the activation of $CGRP_1$ receptors in porcine ciliary arteries. This observation is in agreement with other reports made in the literature on the relaxing effect of adrenomedullin.[14,22]

It has been suggested that in some vascular beds, the effect of adrenomedullin could be mediated by a specific adrenomedullin receptor and the adrenomedullin fragment AM[22-52] has been used as specific adrenomedullin receptor antagonist by some investigators.[15] For example, pretreatment with 100 nM AM[22-52] could attenuate the vasodilation induced by adrenomedullin in human coronary arterioles.[15] In porcine ciliary arteries, we could not confirm this hypothesis, because the relaxation evoked by adrenomedullin was not significantly affected by the adrenomedullin receptor antagonist AM[22-52], even at a concentration as high as 3 μM. Our results are in agreement with other reports conducted in the canine mesenteric arteries and porcine coronary arteries, showing that adrenomedullin-induced relaxation was not affected by AM[22-52].[23,24] It also should be mentioned that the

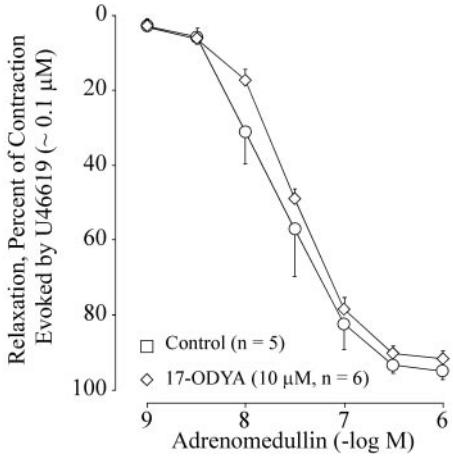

FIGURE 6. Effect of a 17-ODYA, a cytochrome P_{450} inhibitor on adrenomedullin-induced relaxation in isolated porcine ciliary arteries precontracted with the thromboxane A_2 analogue U46619. 17-ODYA had no significant effect on adrenomedullin-induced relaxation.

AM[22-52] fragment is considered by some investigators not to be a very potent adrenomedullin inhibitor, and its specificity has also been questioned.[15,24]

Adrenomedullin has been shown to induce vasodilatation, both through endothelium-dependent[8,9] and -independent mechanisms.[13,25] In this regard, it is interesting to note that heterogeneity can exist between different species or between different vascular beds in the mechanisms involved in adrenomedullin-induced relaxation. For example it has been reported that in canine retinal arteries, adrenomedullin induces only endothelium-independent relaxation.[13] In contrast, in porcine ciliary arteries we demonstrated that the relaxing effect of adrenomedullin is in part endothelium dependent.

Usually, three major factors are considered to be involved in endothelium-dependent relaxation: NO, prostacyclin, and EDHF.[26,27] In the kidneys of the dog or in the hindquarters of the rat, NO has been shown to mediate endothelium-dependent relaxation induced by adrenomedullin.[10,11] In rat pulmonary artery rings in hypoxic conditions, adrenomedullin-induced relaxation was abolished by 10 μM indomethacin, an inhibitor of cyclooxygenase.[9] In the porcine ciliary artery, it appeared that adrenomedullin-induced endothelium-dependent relaxation did not involve NO, because the relaxing effect of this peptide was not significantly affected by L-NAME. Also, prostacyclin did not appear to mediate the relaxing effect of adrenomedullin in porcine ciliary arteries, because the adrenomedullin-induced relaxation was unaffected by indomethacin.

The endothelium-derived factor EDHF relaxes smooth muscle cells through the opening of potassium channels in these cells.[27] The present study shows that in porcine ciliary arteries, the relaxation evoked by adrenomedullin was partially blunted by several inhibitors of potassium channels. The effect of these potassium channel inhibitors was observed only in vessels with a functional endothelium but not in vessels with a mechanically and intentionally damaged nonfunctional endothelium, suggesting the involvement of potassium channels in the endothelium-dependent relaxing effect of adrenomedullin. These observations indicate the possible presence of an EDHF in the endothelium-dependent relaxation evoked by adrenomedullin. These results are in agreement with other reports showing the activation of potassium channels in the adrenomedullin-induced relaxation in rat kidney[28] and human coronary arterioles as well.[12]

The chemical nature and the physiological role of the EDHF are still unclear. It has been proposed that arachidonic acid metabolites produced by the cytochrome P_{450} monooxygenase pathway (epoxyeicosatrienoic acids) may act as an EDHF.[29,30] Indeed, arachidonic acid metabolites have been shown to induce hyperpolarization in arterial smooth muscle cells. It has been reported, for example, that the EDHF-mediated component of an endothelium-dependent relaxation is blunted by inhibitors of cytochrome P_{450}, such as SKF 525A and miconazole in bovine coronary artery[30] and 17-ODYA in rabbit carotid artery.[21] These findings could not be confirmed in other vessels,[31,32] suggesting that different factors may represent EDHF. In our study conducted in isolated porcine ciliary arteries, adrenomedullin-induced relaxation was not significantly affected by the cytochrome P_{450} inhibitor 17-ODYA. Therefore, it is unlikely that a cytochrome P_{450}–derived metabolite of arachidonic acid is involved in the EDHF-induced activation of potassium channels evoked by adrenomedullin in porcine ciliary arteries.

In conclusion, adrenomedullin evoked in porcine ciliary arteries potent relaxation that was in part endothelium dependent and appeared to involve the activation of $CGRP_1$ receptors. Furthermore, the endothelium-dependent component of this relaxation was blocked by some potassium channel inhibitors, suggesting the presence of an EDHF.

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