Pentobarbital-sensitive EDHF comediates ACh-induced arteriolar dilation in the hamster microcirculation

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In studies on large-conductance arteries, ACh mediates independent hyperpolarization of vascular smooth muscle in isolated arteries, whereas ChTX abolished it. This study was without effect. Pentobarbital also reduced the ACh-induced hyperpolarization of vascular smooth muscle in isolated arteries, whereas ChTX abolished it. This study suggests that a considerable part of the ACh dilation in the microcirculation is mediated by EDHF, which also contributes to the control of basal tone in conscious animals. The direct inhibitory effect of pentobarbital and ODYA supports the idea that "microcirculatory" EDHF is a product of the cyclo-oxygenase-P-450 pathway and compared with the P-450-dependent metabolite of arachidonic acid (10). In accordance with this, EDHF-induced dilations of isolated arteries were reduced by various variable concentrations depending on their efficacy to interfere with the cyclo-oxygenase-P-450 pathway (13). As a result, EDHF-mediated dilations in the microcirculation of a conscious animal may differ considerably from those in a barbiturate-anesthetized animal. Some studies demonstrated a NO synthase- and cyclo-oxygenase-independent dilation at the microcirculatory level in anesthetized animals (14, 24), but its relative significance compared with that in conscious animals is rather unclear. Furthermore, the role of EDHF in differently sized arterioles has never been studied systematically in vivo, although in vitro studies suggest that differences may exist between arteries of different calibers (23).

The aim of our study was to investigate EDHF-mediated dilation in vivo and its relative significance in differently sized arterioles. Moreover, we tested which K⁺ channels are involved in EDHF-induced hyperpolarization by the use of specific blocking agents. To elucidate the effect of different anesthetics, EDHF responses were studied in animals before and after the induction of anesthesia with agents exhibiting different potencies to affect the P-450 pathway and compared with the inhibitor of the P-450 monoxygenase, 17-octadecynoic acid (ODYA). Potential cellular mechanisms of the anesthetic pentobarbital on the EDHF-mediated dilations were additionally investigated in isolated arterioles.

METHODS

Preparation of cremaster muscle. Golden Syrian hamsters (80- to 150-g body wt) were anesthetized by intraperitoneal injection of pentobarbital sodium (75 mg/kg) followed by continuous administration of the anesthetic via a jugular vein catheter at a rate of 5–10 mg·kg⁻¹·h⁻¹. Carotid artery pressure was measured continuously by means of a pressure transducer (Statham, Costa Mesa, CA), and data were stored on computer disk. The animals were artificially ventilated (7025 Rodent Ventilator, Hugo Sachs Elektronik, Freiburg, Germany) to maintain Po2 and Pco2 at physiological values (~100 and 40 mmHg, respectively), as determined by blood gas analysis. The right cremaster muscle was prepared as previously described (27). The care of the animals and the...
conduct of the experiments were in accordance with the rules of the German animal protection laws.

Preparation of dorsal skinfold chamber. Arteriolar responses in conscious hamsters were studied using the dorsal skinfold chamber, containing the thin striated skin muscle within an observation window. Implantation of the chambers was performed under pentobarbital anesthesia as described previously (12). One epidermal layer was completely removed to expose the underlying skin muscle, which was thereafter protected by a coverslip. The animals were then allowed to wake up, and a period of 72–96 h was allowed before investigation of the microcirculation to eliminate the effects of anesthesia and surgical trauma. For local application of vasoactive substances, the coverslip was removed and the skin muscle was superfused as described in Experimental Protocols. In this way, arterioles could be monitored without the use of fluorescent dyes.

Experimental setup. The muscle was superfused with warm (34°C) bicarbonate-buffered salt solution at a rate of 8 ml/min. The superfusion fluid had a pH of 7.4, a Po2 of ~30 mmHg, and a PCO2 of ~38 mmHg as measured in samples taken from the surface of the muscle. One or two arterioles were studied in each animal. Arterioles studied in different animals were of varying size and vascular generation and were monitored by means of a microscope (Metallux, Leitz, Wetzlar, Germany) equipped with a video camera. If more than one arteriole was studied, the other vessels were investigated subsequently, repeating the same protocol. The microscopic images were displayed on a video monitor at 720-fold magnification and recorded on videotape (S-VHS, Sony). Arteriolar inner diameters were measured off-line from digitized images (MVP-AT, Matrox, Dorval, PQ, Canada) using a laboratory computer program.

Experimental protocols. After the hamsters were placed onto a microscope stage, the preparations were allowed to recover for 30 min before the start of the experiments. The vascular diameter of an arteriole was measured before (1–2 min) and during the local superfusion (3–4 min) of the endothelium-independent NO donor sodium nitroprusside (1 µmol/l) and during the local superfusion (3–4 min) of the endothelium-dependent NO donor L-arginine (100 µmol/l). The arteriolar responses were studied before and after addition of L-NNA (100 µmol/l), indomethacin (10 µmol/l), and papaverine (300 µmol/l), the responses on addition of ACh or SNP were investigated again. In two separate groups of conscious hamsters pretreated with L-NNA and indomethacin, arteriolar responses were studied before and after charybdotoxin (1 µmol/l) or ODYA (50 µmol/l). The effects of the Ca2+-channel blocker glibenclamide (1 µmol/l) and the ATP-dependent Na+450 monooxygenase, ODYA, were added to the superfusion fluid for 15 min and washed out before reexamination of the arteriolar responses to ACh and SNP. To determine the maximal diameter of each vessel, a combination of the vasodilators adenosine (100 µmol/l), SNP (10 µmol/l), and papaverine (300 µmol/l) was applied at the end of the experiment, and the resulting maximal diameter was measured.

Preparation of isolated arteries. Female golden Syrian hamsters (120- to 140-g body wt) were anesthetized by intraperitoneal injection of pentobarbital. The method for the isolation of a small artery (diameter 160 µm) and mounting of intracellular calcium and diameter was described in detail elsewhere (2). Briefly, the right femoral artery was quickly exposed and occluded proximal to the site of the isolation of a microvessel, thereby preventing its exposure to the high concentration of pentobarbital, which was used to kill the animal later. The isolated small artery was cannulated, pressurized (45 mmHg), and loaded with fura 2-AM (MOPS-buffered salt solution, 2 µmol/l fura 2-AM, 0.5% bovine serum albumin) or the potential-sensitive dye bisoxonol [bis(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC4(3)); 50 µmol/l]. Intracellular free calcium ([Ca2+]i) or membrane potential changes and diameter were measured by means of a modified inverted microscope (Olympus IMT-2) equipped with a ×20 lens (Olympus D-AP0 20 UV) and a video camera system. Alternating excitation wavelengths of 340 and 380 nm were used for measurement of [Ca2+]i, and the fluorescence at a wavelength of 510 nm was recorded using a photomultiplier tube (Photomulti, Wiesbaden, Germany). The ratio of fluorescence at 340 and 380 nm was calculated after subtraction of the background fluorescence (obtained after fura 2 quenching with 8 mmol/l MnCl2). For membrane potential measurement, the dye was excited at 490 nm and the fluorescence was recorded at 516 nm. Additionally, the vessel was transilluminated at wavelengths >610 nm, which did not interfere with the fluorescence measurements, and diameter was measured as described above.

Protocol in isolated arteries. The vessels were allowed to develop spontaneous myogenic tone in response to the applied transmural pressure for 30 min before the start of the experiments, which were carried out in the presence of indomethacin (30 µmol/l). All vessels were preconstricted with norepinephrine (NE, 0.3 µmol/l) 2 min before the addition of ACh (1 µmol/l). This was done in the same vessels (n = 4) before and 10 min after addition of pentobarbital (1–2 mM) to the organ bath. The changes in membrane potential induced by ACh were measured in a separate group of vessels (n = 6) and repeated after addition of charybdotoxin (1 µmol/l) or pentobarbital (2 mM) to the organ bath.

Solutions and drugs. The salt buffer used for superfusion was of the following composition (in mmol/l): 143 Na+, 6 K+, 2.5 Ca2+, 1.2 Mg2+, 128 Cl−, 25 HCO3−, 1.2 SO4−, and 1.2 H2PO4−. The MOPS-buffered salt solution used for experiments on isolated vessels consisted of (in mmol/l) 145 NaCl, 30 MOPS, 11.5 KCl, 1.4 CaCl2, 0.8 MgSO4, 4.7 NaHCO3, 1.2 KH2PO4. The Krebs-Ringer solution contained (in mmol/l) 146 NaCl, 25 KCl, 1.2 CaCl2, 1.2 MgCl2, 1.2 NaHCO3, 11.5 glucose, and 0.5 triethanolamine. All drugs were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.
4.7 KCl, 1.5 CaCl2, 1.17 MgSO4, 1.2 NaH2PO4, 2.0 pyruvate, 0.02 EGTA, 3.0 MOPS, and 5.0 glucose. ACh, SNP, NE, ODYA, and charybdotoxin were purchased from Sigma (Deisenhofen, Germany); L-NNa from Serva (Heidelberg, Germany), indomethacin (Confortid) from Dumex (Bad Vivel, Germany), fura 2-AM and DiBAC4(3) from Molecular Probes, and MnCl2 from Merck (Darmstadt, Germany). Felo dinpine was a kind gift of Astra (Wedel, Germany). Stock solutions of indomethacin (12 mmol/l), furo (1 mmol/l), and fura 2-AM (1 mmol/l) were prepared in pure water, ethanol, or water-free dimethyl sulfoxide, respectively, and stored at -20°C until use. On the day of the experiment, SNP (10 mmol/l) was dissolved in 1 mmol/l Na-acetate and L-NNa was dissolved in a HEPES-buffered salt solution by vigorous stirring at 60°C for 30 min. For all other solutions and further dilutions, freshly prepared superfusion buffer was used. All locally applied drugs (concentrated 100-fold over the final concentration) were added to the superfusion fluid by means of a roller pump at 1/100th of the total superfusion rate (0.08 ml/min) to obtain the final concentrations indicated above except for charybdotoxin (see Experimental Protocols).

Statistics and calculations. Vascular tone is expressed as the quotient of vessel resting diameter divided by its maximal diameter. Changes in inner diameter of the vessels were normalized to the maximal possible constriction or dilation according to the relationship

\[
\text{% of maximal response} = \frac{(D_{r} - D_{C})}{(D_{M} - D_{C})} \times 100
\]

where \(D_{r}\) represents the diameter observed after treatment and \(D_{C}\) the control diameter before treatment. \(D_{r}\) represents (for dilator responses) the diameter at maximal dilation or (for constrictions) the minimal inner diameter (zero). This normalization enables the comparison of vessels of different tone and size.

In isolated vessels, diameters and calcium and membrane potential values are expressed as percent change

\[
\text{% change} = \frac{|(V_{r} - V_{C})|}{V_{C}} \times 100 - 100
\]

where \(V_{r}\) represents the value after treatment and \(V_{C}\) the steady state value of the NE-preconstricted vessel. Because of potential errors associated with [Ca2+] measurements in intact vessels (17), we decided to use fluorescence ratio values to calculate percent changes of [Ca2+]. According to calibrations in a cell-free system, the range of ratios observed here (0.65–1.7) fitted well into the linear range of the calibration curve (42.2–1,520 nmol/l), which is a prerequisite for calculating percent changes.

Comparisons within groups were performed using paired t-tests and, for multiple comparisons, were corrected according to Bonferroni. Values between groups were compared by analysis of variance followed by post hoc analysis of the means. Differences were considered significant at a corrected error probability \(P < 0.05\).

RESULTS

Arteriolar dilations in cremaster muscle of anesthetized animals. A total of 28 arterioles with maximal luminal diameters between 34 and 99 µm (mean: 57.6 ± 3.1 µm) were studied in 24 animals. Arterial blood pressure was 86 ± 4 mmHg at the start of the experiment and was virtually stable throughout the experiment (end: 91 ± 4 mmHg, \(P = 0.42\)). Similarly, heart rate did not vary significantly during the experiment (305 ± 14 beats/min). The vessels exhibited varying degrees of spontaneous tone, i.e., the quotient of resting to maximal diameter ranged from 0.37 to 0.68 (mean: 0.53 ± 0.02). Local superfusion of 10 and 100 µmol/l ACh dilated the arterioles by 59.5 ± 6.2 and 75.0 ± 5.1%, respectively. The dilation on ACh superfusion lasted as long as ACh was applied (4 min) without an attenuation of the dilation during this period of observation (Fig. 1). The addition of L-NNa and indomethacin reduced arteriolar diameters from 28.9 ± 1.6 to 23.9 ± 1.8 µm (−18.1 ± 3.8%) and led to a significant attenuation of the ACh-induced dilation. However, ACh still induced a significant dilation (Fig. 1). This L-NNa- and indomethacin-resistant dilation decreased with time after ACh application (1 vs. 3 min: 29.9 ± 4.8 vs. 17.1 ± 4.2% at 10 µmol/l, \(P < 0.05\); 43.6 ± 4.6 vs. 30.1 ± 5.9% at 100 µmol/l, \(P = 0.09\)). However, a significant dilation was found even after 4 min (Fig. 1). Under control conditions as well as in the presence of L-NNa and indomethacin the normalized ACh-induced
dilations were of a similar magnitude in small (maximal diameter <50 µm) and large (maximal diameter >50 µm) arterioles (Table 1).

The elevation of the extracellular K+ concentration ([K+]e; 50 mmol/l) induced a strong constriction of the arterioles (data not shown). To prevent this constriction induced by the accompanying depolarization, the addition of K+ to the superfusion buffer was combined with the L-type Ca2+-channel blocker felodipine (1 µmol/l). Vascular diameters remained unaffected by this treatment (23.8 ± 2.7 vs. 24.4 ± 2.2 µm; P = 0.79) despite the elevated [K+]e. When applied without the elevation of [K+]e, felodipine induced a small vasodilation (from 17.4 ± 2.0 to 20.7 ± 1.5 µm; P < 0.05). Although felodipine alone did not attenuate the L-NNA- and indomethacin-resistant dilation on ACh administration (10 µmol/l; 24.0 ± 7.9 vs. 18.5 ± 4.8%, P = 0.57, n = 5; 100 µmol/l; 49.0 ± 10.6 vs. 53.4 ± 15.3%, P = 0.89, n = 5), elevated [K+]e abolished the ACh dilation (Fig. 1). Neither L-NNA in combination with indomethacin nor the elevation of [K+]e significantly attenuated the dilation elicited by the NO donor SNP (Table 2). To evaluate the type of K+ channel involved in the dilation we applied specific inhibitors of K+ channels. Glibenclamide (1 µmol/l), a blocker of ATP-dependent K+ channels, affected neither the vascular resting diameter nor the L-NNA- and indomethacin-independent dilation induced by ACh (Fig. 2). However, charybdotoxin (1 µmol/l), a blocker of large-conductance Ca2+-dependent K+ channels, abolished the L-NNA- and indomethacin-resistant portion of the ACh-induced dilation (Fig. 2). The dilation on SNP application, on the other hand, was not affected by charybdotoxin (Fig. 2). The application of charybdotoxin did not change the arteriolar resting diameter (25.3 ± 3.8 vs. 26.4 ± 3.7 µm; P = 0.37).

Effect of anesthesia, ODYA, and charybdotoxin on arteriolar dilations in skin muscle. Twenty-three arterioles with maximal luminal diameters between 52 and 125 µm (mean: 76.4 ± 3.5 µm) were studied in the skin muscle of 23 conscious animals. The spontaneous resting tone of these vessels ranged from 0.39 to 0.85 (mean: 0.66 ± 0.02), L-NNA and indomethacin reduced the arteriolar diameter from 56.5 ± 3.1 to 47.7 ± 3.2 µm (12.0 ± 5.3%) and the dilation on application of ACh (10 µmol/l): 56.2 ± 4.8 vs. 33.4 ± 3.2%, P < 0.05; 100 µmol/l: 68.5 ± 4.3 vs. 47.9 ± 4.6%, P < 0.05), whereas the dilation in response to SNP (10 µmol/l) remained unaffected (64.2 ± 3.8 vs. 63.3 ± 3.8%, P = 0.87, n = 21). The induction of anesthesia in the animals by pentobarbital itself did not alter basal diameters (44.9 ± 7.9 vs. 51.5 ± 8.3 µm; P = 0.08) but led to a significant attenuation of the L-NNA- and indomethacin-resistant portion of the ACh dilation (Fig. 3). This pentobarbital effect was specific for ACh, because the dilation on SNP application remained unaffected (57.1 ± 7.1 vs. 59.6 ± 6.7%, P = 0.67, n = 7). In contrast, if urethan was used to anesthetize different hamsters (n = 4), the dilation in response to ACh remained unaltered (Fig. 3). Anesthesia induction using urethan also did not alter the arteriolar resting diameter (56.0 ± 5.2 vs. 54.2 ± 5.0 µm) or the dilation on application of SNP (data not shown).

In two separate groups of conscious animals, the effects of an inhibitor of the P-450 monooxygenase, ODYA, as well as a blocker of Ca2+-dependent K+ channels, charybdotoxin, on the L-NNA- and indomethacin-resistant ACh dilation were investigated. ODYA (50 µmol/l) reduced these dilations to a similar degree

Table 1. Vascular dilations induced by acetylcholine in small and large arterioles

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<tr>
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<th>Large Arterioles</th>
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<th>Small Arterioles</th>
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<tr>
<td></td>
<td>Diameter, µm</td>
<td>Dilation, %</td>
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<td>n</td>
<td>Before</td>
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<td>ACh (10 µmol/l)</td>
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<tr>
<td>Control</td>
<td>13</td>
<td>33.6 ± 1.7</td>
<td>55.5 ± 4.0</td>
<td>65.0 ± 7.9</td>
</tr>
<tr>
<td>L-NNA + Indo</td>
<td>10</td>
<td>25.5 ± 2.2</td>
<td>33.9 ± 4.1</td>
<td>22.7 ± 6.5</td>
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<tr>
<td>+[K+]e</td>
<td>6</td>
<td>28.1 ± 2.9</td>
<td>26.2 ± 3.2</td>
<td>−7.0 ± 4.8</td>
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<tr>
<td>ACh (100 µmol/l)</td>
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<tr>
<td>Control</td>
<td>8</td>
<td>28.7 ± 2.5</td>
<td>55.0 ± 4.0</td>
<td>82.7 ± 3.6</td>
</tr>
<tr>
<td>L-NNA + Indo</td>
<td>10</td>
<td>27.3 ± 2.7</td>
<td>39.5 ± 3.6</td>
<td>37.1 ± 8.2</td>
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<tr>
<td>+[K+]e</td>
<td>7</td>
<td>26.4 ± 2.3</td>
<td>27.1 ± 2.5</td>
<td>1.1 ± 3.9</td>
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Values were obtained in 24 animals and are presented as means ± SE; n, no. of arterioles. Dilations are given as % of maximal response as obtained 2 min after addition of ACh to superfusion buffer. L-NNA, N altered nitro-L-arginine (30 µmol/l); Indo, indomethacin (3 µmol/l); +[K+]e, extracellular K+ concentration was elevated to 50 mmol/l by exchanging K+ for Na+.

Table 2. Vascular dilations induced by SNP under control conditions and after L-NNA + indomethacin or additional elevation of [K+]e

<table>
<thead>
<tr>
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<th>SNP</th>
<th>1 µmol/l</th>
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<td>n</td>
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<tr>
<td>Control</td>
<td>18</td>
<td>43.0 ± 6.3</td>
<td>10</td>
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<tr>
<td>L-NNA (30 µmol/l)</td>
<td>15</td>
<td>51.4 ± 3.6</td>
<td>14</td>
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<tr>
<td>+ Indo (3 µmol/l)</td>
<td>15</td>
<td>39.8 ± 9.5</td>
<td>8</td>
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</table>

Values were obtained in 20 animals and are presented as means ± SE; n, no. of arterioles. Dilations are given as % of maximal response as obtained 2 min after addition of sodium nitroprusside (SNP) to superfusion buffer. Diameter changes were not significantly different between treatments (ANOVA).
as found by pentobarbital anesthesia, whereas charybdotoxin (1 µmol/l) abolished them (Fig. 3). Neither ODYA [64.3 ± 11.3 vs. 58.2 ± 9.9%, P = not significant (NS)] nor charybdotoxin (68.8 ± 4.9 vs. 71.4 ± 4.1%, P = NS) altered the dilations induced by SNP (10 µmol/l). In contrast to the results in pentobarbital-anesthetized animals, charybdotoxin reduced the arteriolar resting diameter from 37.1 ± 2.9 to 33.3 ± 3.4 µm (−10.2 ± 3.8%; P < 0.05) in conscious animals.

Fig. 2. Effect of specific K⁺ channel blockers on ACh [10 (A) and 100 (B) µmol/l] and sodium nitroprusside [SNP, 10 µmol/l (C)]-induced dilations. In presence of L-NNA + Indo, glibenclamide (Gb) did not significantly affect dilations on ACh or SNP application, whereas charybdotoxin (ChTX) virtually abolished ACh dilations. In contrast, dilations induced by nitric oxide (NO) donor SNP were not affected by ChTX. Dilation is expressed as % of maximal response. Resting (r) and maximal (m) vessel diameters (in µm) are as follows. In Gb groups (n = 3), ACh (10 µmol/l): L-NNA + Indo 21.1 ± 3.8 (r), + Gb 22.3 ± 5.3 (r); ACh (100 µmol/l): L-NNA + Indo 21.0 ± 3.5 (r), + Gb 19.8 ± 4.8 (r); SNP (10 µmol/l): L-NNA + Indo 17.7 ± 1.1 (r), + Gb 20.3 ± 5.0 (r) and 43.2 ± 2.7 (m). In ChTX groups, ACh (10 µmol/l): L-NNA + Indo (n = 4) 23.1 ± 3.8 (r) and 60.2 ± 3.1 (m), + ChTX (n = 5) 29.7 ± 3.7 (r) and 62.3 ± 3.2 (m); ACh (100 µmol/l): L-NNA + Indo (n = 3) 25.3 ± 4.0 (r) and 62.0 ± 4.9 (m), + ChTX (n = 6) 25.9 ± 3.1 (r) and 60.5 ± 5.6 (m); SNP (10 µmol/l): L-NNA + Indo (n = 7) 17.3 ± 2.8 (r) and 57.1 ± 0.5 (m), + ChTX (n = 6) 26.7 ± 3.4 (r) and 58.8 ± 4.4 (m). Values were obtained in 3 (Gb) and 4 (ChTX) animals. *P < 0.05 vs. respective control response (paired t-test).

Fig. 3. Effect of anesthesia, 17-octadecynoic acid (ODYA), and ChTX on ACh dilations in skin muscle. NO- and prostaglandin-independent vasodilation induced by ACh [10 (A) and 100 (B) µmol/l] in skin muscle of conscious hamsters was attenuated after pentobarbital but not urethan anesthesia. In conscious animals, ODYA (50 µmol/l, n = 5) reduced ACh dilation to a similar degree as did pentobarbital, whereas blocker of Ca²⁺-dependent K⁺ channels ChTX (n = 6) abolished it. Resting (r) and maximal (m) vessel diameters (in µm) are as follows. Pentobarbital (n = 7): 49.1 ± 7.6 (r), 80.6 ± 8.7 (m); urethan (n = 4): 53.7 ± 4.0 (r), 77.1 ± 1.8 (m); ODYA (n = 5): 53.9 ± 4.9 (r), 82.2 ± 5.8 (m); ChTX (n = 5): 40.2 ± 4.7 (r), 67.9 ± 7.5 (m). All data were obtained in presence of L-NNA and Indo. *Significant differences (P < 0.05, paired t-test) between treatment and respective control response.
ACh-induced \([\text{Ca}^{2+}]_i\) decrease was significantly attenuated and the diameter increase tended to be smaller. When a higher concentration of pentobarbital was used (2 mmol/l), the ACh-induced \([\text{Ca}^{2+}]_i\) reduction was virtually abolished and the concomitant relaxation was significantly attenuated (Fig. 4). Similar observations were found in this model after blockade of \([\text{Ca}^{2+}]_i\)-dependent \(K^+\)-channel blocker charybdotoxin (data not shown). In different arteries loaded with the potential-sensitive dye bisoxonol, ACh hyperpolarized vascular smooth muscle cells as reflected by a 12 ± 1% reduction of the fluorescence signal. This hyperpolarization was reduced in the presence of pentobarbital (Fig. 5) and virtually abolished in the presence of charybdotoxin (−4 ± 2% of the fluorescence signal, n = 4).

**DISCUSSION**

These experiments support the hypothesis that ACh-induced dilation in the microcirculation is partially an EDHF-mediated response. This view is substantiated by the fact that the dilation was abolished by an elevation of \([K^+]_e\) or by the \([\text{Ca}^{2+}]_i\)-dependent \(K^+\)-channel blocker charybdotoxin, which also abolished the ACh-induced hyperpolarization of vascular smooth muscle in isolated vessels. Of note, this NO- and prostaglandin-independent dilation was partially sustained in the continuous presence of ACh, which is in contrast to most in vitro findings. The dilation was strongly attenuated by ODYA, a blocker of the cytochrome P-450 pathway, and by anesthesia with pentobarbital but not with urethan, suggesting that microcirculatory EDHF is a cytochrome P-450-related metabolite.

In conscious as well as anesthetized animals a major part of the arteriolar dilation induced by ACh was mediated by NO and/or prostanoids. This is in accordance with a previous study using the same model (27). In that study using ACh concentrations up to 1 µmol/l, only a small fraction of the dilation was resistant to NO and cyclooxygenase blockade. Because the skeletal muscle that surrounds the arterioles contains high acetylcholinesterase activity, the concentration (1 µmol/l) used in that study was obviously too low to induce the release of substantial amounts of EDHF. This assumption is supported by observations in isolated hamster arterioles and in other tissues in which the stimulation of EDHF release required higher concentrations of ACh than the stimulation of NO release (2). This difference might be related to different receptor types involved in the release of the respective endothelial dilator. For instance, different types of muscarinic receptors exhibit striking differences in their respective EC\(_{50}\) (11). As shown in the present study, high concentrations of ACh induced a dilation that could not be prevented by NO synthase and cyclooxygenase inhibitors but was completely blocked by the elevation of \([K^+]_e\) (50 mmol/l) (Fig. 1). In
contrast, dilations induced by the NO donor SNP remained unaffected by high potassium (Table 2). The divergent inhibitory potency of the elevation of [K\textsuperscript{+}]\textsubscript{e} excludes the possibility that blockade of the NO synthase was insufficient at higher concentrations of ACh. Moreover, the inhibitory effect of the elevated [K\textsuperscript{+}]\textsubscript{e} suggests that the remaining part of the ACh dilation was caused by a hyperpolarization accomplished by the activation of K\textsuperscript{+} channels. In fact, ACh induced a hyperpolarization of vascular smooth muscle cells in isolated, preconstricted arteries.

The findings of our study are in agreement with several studies on conductance arteries of different vascular beds and species. In most of these studies a L-NNA- and indomethacin-resistant dilation was demonstrated, which was mediated by hyperpolarization (8, 15) as confirmed by electrophysiological studies (16, 22). Different types of K\textsuperscript{+} channels have been identified in vascular smooth muscle. Data obtained on small arteries or arterioles in vitro suggest that the type of the K\textsuperscript{+} channel involved seems to vary depending on the vascular bed and species (6, 20, 30). Because charybdotoxin, but not glibenclamide, abolished the NO synthase- and cyclooxygenase-independent dilation (Figs. 2 and 3) as well as the ACh-induced hyperpolarization, our study supports the concept that Ca\textsuperscript{2+}- dependent, but not ATP-dependent, K\textsuperscript{+} channels are involved in the dilation mediated by EDHF, at least in the hamster microcirculation.

It must be noted, however, that the design of this study (microcirculation in vivo) did not allow differentiation between K\textsuperscript{+} channels located in the vascular smooth muscle and those located in the endothelium. In the case of the latter, endothelial hyperpolarization induced by the activation of endothelial K\textsuperscript{+} channels could affect the synthesis or release of a potential EDHF. Furthermore, endothelial hyperpolarization could be a modulator of myoendothelial signal transmission, which has recently been proposed to underlie “EDHF”-dependent dilation (5). However, the endothelial Ca\textsuperscript{2+} increase on application of ACh remained unaffected by charybdotoxin (S. S. Bolz, unpublished observation). This supports the view that charybdotoxin does not act on the endothelium but rather on the vascular smooth muscle cell.

It has been suggested that EDHF-mediated dilations are of a greater magnitude in smaller arteries (23) and that the role of EDHF could be more important in those vessels further downstream. This hypothesis cannot be extrapolated to the even smaller vessels of the microcirculation. As shown in this study, the L-NNA- and indomethacin-resistant dilation was of a similar magnitude in small (diameter \(<50 \mu m\)) and large (diameter \(>50 \mu m\)) arterioles.

To prevent the vasoconstriction induced by depolarization (caused by elevation of [K\textsuperscript{+}]\textsubscript{e}), voltage-dependent L-type Ca\textsuperscript{2+} channels were blocked by felodipine (9). It can be assumed that the concentration used (1 \(\mu mol/l\)) is sufficient to block these channels, because the resting diameter increased on addition of felodipine. Moreover, addition of felodipine abolished the constriction induced by the elevated K\textsuperscript{+} concentration. Felodipine might act as a calmodulin inhibitor (25). Therefore, the blockade of the L-NNA- and indomethacin-resistant dilations in the presence of elevated [K\textsuperscript{+}]\textsubscript{e} might be attributed to a calmodulin-inhibiting effect of the concomitantly applied felodipine. A direct inhibitory effect of felodipine on the release or action of the putative EDHF can, however, be excluded because felodipine alone did not abolish the L-NNA- and indomethacin-resistant dilation induced by ACh. Rather, the elevation of [K\textsuperscript{+}]\textsubscript{e} was necessary to exert the blockade. It has been proposed that a hyperpolarization (induced by EDHF) elicits vasodilation by closing voltage-dependent L-type Ca\textsuperscript{2+} channels (19). The fact that the dilation remained unaffected by the L-type Ca\textsuperscript{2+}-channel blocker suggests that EDHF exerts a dilation independent of these Ca\textsuperscript{2+} channels. Alternative mechanisms by which a hyperpolarization might exert vasodilation have been proposed (7), e.g., the interaction with the synthesis of inositol 1,4,5-trisphosphate (29). Nevertheless, the action of EDHF depends critically on the hyperpolarization induced by the activation of K\textsuperscript{+} channels.

Studies performed in the coronary vascular bed of the rat (1) and porcine and bovine arteries (4, 10, 26) suggest that EDHF is a metabolite of the cytochrome P-450 pathway. In accordance with this, the action and/or synthesis of this factor is attenuated by certain barbiturates (e.g., thiopental) (13). Our comparison of ACh responses in conscious and anesthetized animals supports these observations. The L-NNA- and indomethacin-resistant dilation in conscious animals was significantly attenuated by ODYA, a relatively specific blocker of the P-450 pathway. Pentobarbital anesthesia depressed this part of the ACh dilation to a similar degree (Fig. 3). In contrast, the dilations in response to an exogenous NO donor were not attenuated by pentobarbital, which excludes a nonspecific effect on the vascular smooth muscle function. This effect of pentobarbital is not caused by general anesthesia per se, because urethan did not affect the L-NNA- and indomethacin-resistant portion of the ACh dilation. The additional data obtained in isolated microvessels (Fig. 4) further confirm that pentobarbital blocks EDHF-mediated dilations at the vascular level. The rapid decrease of [Ca\textsuperscript{2+}], the hyperpolarization of the vascular smooth muscle cell, as well as the concomitant rapid dilation of the arteries are typical for EDHF responses in these vessels (2). All these responses were strongly attenuated by pentobarbital and virtually abolished by charybdotoxin. Because the effect of pentobarbital was found in vivo as well as in isolated vessels, we conclude that the attenuation of the putative EDHF release and/or action is caused by a direct effect of the compound. Moreover, because pentobarbital has the potential to inhibit cytochrome P-450 monooxygenase (21) and ODYA exhibited a similar pattern of inhibition, it might be speculated that EDHF is in the microcirculation, similar to larger arteries, a metabolite of the cytochrome P-450 pathway.
In conclusion, our data are consistent with the concept that ACh-induced dilation in the microcirculation is partially caused by a hyperpolarization, which is elicited by an EDHF activating charybdotoxin-sensitive K⁺ channels. Its role may be underestimated in studies on barbiturate-anesthetized animals because its action or, even more likely, synthesis is attenuated by pentobarbital. EDHF seems also to contribute to the control of the vessel’s resting tone. This contribution is solely observed in conscious animals because charybdotoxin constricts the arterioles only in the absence of pentobarbital. The inhibitory effects of ODYA and pentobarbital strongly suggest that “microcirculatory” EDHF is a product of the cytochrome P-450 pathway.

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