Evidence against the involvement of cytochrome P450 metabolites in endothelium-dependent hyperpolarization of the rat main mesenteric artery

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1. The influence of different inhibitors of cytochrome P450 mono-oxygenase on the endothelium-dependent and -independent hyperpolarization in the isolated rat main mesenteric artery was investigated.

2. Application of acetylcholine (ACh; 1 μM) for 10 min evoked an endothelium-dependent peak hyperpolarization of about 18 mV followed by a partial recovery to a level 7 mV more negative than the resting value ($-50{\cdot}2 \pm 0{\cdot}5$ mV).

3. Proadifen (30 μM) completely and reversibly inhibited the ACh-induced hyperpolarization. Conversely, the imidazole antimycotics clotrimazole (30 μM) and miconazole (100 μM) had less effect on the peak endothelium-dependent hyperpolarization. The suicide substrate inhibitors 17-octadecynoic acid (17-ODYA; 5 μM) and 1-aminobenzotriazole (1-ABT; 2 mM) did not significantly influence endothelium-dependent hyperpolarization.

4. The endothelium-independent hyperpolarization (16 mV) evoked by levcromakalim (300 nM) was completely inhibited by proadifen as well as by clotrimazole and miconazole but was not affected by 17-ODYA or 1-ABT.

5. These results do not support the view that the ACh-induced endothelium-dependent hyperpolarization in the rat mesenteric artery is mediated by cytochrome P450 mono-oxygenase metabolites. Proadifen and imidazole antimycotics impair the activation of ATP-regulated K^+ channels in mesenteric artery cells, rendering non-specific inhibition of smooth muscle K^+ channel activation an alternative explanation for the inhibitory influence of some (but not all) P450 inhibitors on endothelium-dependent hyperpolarization in this preparation.

Acetylcholine (ACh) relaxes vascular smooth muscle by stimulating the endothelial cells to release various diffusible substances (Furchgott & Vanhoutte, 1989). Endothelium-derived nitric oxide (NO) is synthesized from L-arginine by the constitutive NO synthase, which can be inhibited by arginine analogues such as nitro-L-arginine (Rees, Palmer, Schultz, Hodson & Moncada, 1990). NO diffuses to the underlying vascular smooth muscle and activates soluble guanylyl cyclase, leading to increased muscular cGMP levels and relaxation (Rapoport & Murad, 1983; Ignarro & Kadowitz, 1985). In some vessels under certain conditions, NO also causes hyperpolarization of the vascular smooth muscle cells (Tare, Parkington, Coleman, Neild & Dusting, 1990; Garland & McPherson, 1992; Vanheel, Van de Voorde & Leusen, 1994). In addition, in some vascular tissues prostacyclin released by the endothelial cells produces a slowly developing and limited hyperpolarization (Parkington, Tare, Tonta & Coleman, 1993; Murphy & Brayden, 1995). However, liberation of a distinct substance, the endothelium-derived hyperpolarizing factor (EDHF), is responsible for the main and often single component of hyperpolarization in response to ACh and other vasoactive agonists (Chen, Suzuki & Weston, 1988; Taylor & Weston, 1988; Félétou & Vanhoutte, 1988; Vanheel *et al.* 1994). The EDHF-induced hyperpolarization results from an increase in K^+ conductance of the smooth muscle cell membrane (Chen & Suzuki, 1989; Fujii *et al.* 1992). The nature of the K^+ channels involved is not yet established. In several arteries, including the main mesenteric artery (Fujii *et al.* 1992) and its smaller branches (McPherson & Angus, 1991; Garland & McPherson, 1992), the EDHF-induced hyperpolarization is not sensitive to glibenclamide, the sulphonylurea which selectively inhibits the activation of ATP-regulated K^+ (K_{ATP}) channels by K^+ channel openers such as levcromakalim. The inhibitory influence of low concentrations of tetraethylammonium (TEA; 3 mM) or tetrabutylammonium (TBA; 1 mM) rather suggests the involvement of Ca^{2+}-dependent K^+ (K_{Ca}) channels (Van de Voorde, Vanheel & Leusen, 1992; Zygmunt & Högestätt, 1996).

The chemical nature of EDHF is as yet unknown. Some studies, in which the nitro-L-arginine-resistant component of vasodilatation was measured in bovine, porcine and rat

coronary arteries, suggest that the substance liberated by the endothelium in response to bradykinin might be a metabolite of arachidonic acid, derived from cytochrome P450-dependent mono-oxygenase activity (Bauersachs, Hecker & Busse, 1994; Hecker, Bara, Bauersachs & Busse, 1994). Epoxyeicosatrienoic acids (EETs) indeed have a potent vasorelaxant action and have been shown to hyperpolarize bovine coronary smooth muscle cells (Campbell, Gebremedhin, Pratt & Harder, 1996) and increase K_{Ca} channel activity in smooth muscle cells from several arteries (Gebremedhin, Ma, Falck, Roman, Vanrollins & Harder, 1992; Hu & Kim, 1993; Campbell *et al.* 1996).

The aim of the present study was to investigate the role of cytochrome P450-dependent arachidonic acid metabolism in endothelium-dependent hyperpolarization elicited by ACh in the rat main mesenteric artery. After characterization of the hyperpolarization induced by EDHF, we used quinacrine, which blocks phospholipase A_2-induced liberation of arachidonic acid, and five structurally and mechanistically different cytochrome P450 inhibitors: proadifen, clotrimazole, miconazole, 17-octadecynoic acid (17-ODYA) and 1-aminobenzotriazole (1-ABT).

METHODS

Preparation

The mesentery from 4- to 6-week-old Wistar rats, anaesthetized with an intraperitoneal injection of a lethal dose (200 mg kg^{-1}) of pentobarbitone, was excised and placed in cold normal Krebs–Ringer solution of the following composition (mmol l^{-1}): NaCl, 135; KCl, 5; $NaHCO_3$, 20; $CaCl_2$, 2·5; $MgSO_4.7H_2O$, 1·3; KH_2PO_4, 1·2; EDTA, 0·026; and glucose, 10. The solution was gassed with a 95% O_2–5% CO_2 gas mixture. The superior mesenteric artery was dissected free of adherent connective tissue and cut into 4–6 mm long ring segments which were subsequently slit along their longitudinal axis. For membrane potential (V_m) measurements, a strip was pinned down, luminal side upwards, to the bottom of a small recording chamber (35 °C), which was continuously perfused (3 bath volumes per minute) with warmed (35 °C) and oxygenated Krebs–Ringer fluid of pH 7·4. The preparation was equilibrated for at least 60 min before the start of the microelectrode impalements.

Electrophysiological measurements

Transmembrane potentials were measured using conventional microelectrodes, pulled with a vertical pipette puller (David Kopf, model 750; Tujunga, CA, USA) from 1 mm o.d. filamented borosilicate glass tubing (Hilgenberg GmbH, Malsfeld, Germany). Microelectrodes were filled with 1 M KCl. The electrical resistance of the microelectrodes ranged from 40 to 80 MΩ. The measured potential was followed on an oscilloscope and traced with a pen recorder (Philips PM 8224). Absolute values of V_m were taken as the difference of the stabilized potential after cell impalement and the zero potential upon withdrawal of the microelectrode from the cell. Hyperpolarizations produced by 10 min applications of either ACh or levcromakalim under control conditions and after experimental intervention were only measured from continuous recordings. Moreover, we usually compared the changes in V_m under control conditions and after experimental intervention from continuous traces obtained during the same cell impalement. The various inhibitors were superfused for at least 10 min before challenging the preparation with the hyperpolarizing vasodilators. Since repeated applications of ACh elicited progressively decreasing V_m responses (see Results), we always tried to measure, in addition, the hyperpolarization after washout of the various inhibitors.

Drugs

Acetylcholine chloride, tetraethylammonium chloride, indomethacin, quinacrine, proadifen (SKF525a), clotrimazole, miconazole nitrate, 17-ODYA and 1-ABT were obtained from Sigma. Glibenclamide was obtained from Merck. Levcromakalim (BRL 38227) was a gift from Beecham Pharmaceuticals, Essex, UK. Stock solutions were made in water, except for acetylcholine chloride (dissolved in 50 mM potassium hydrogen phthalate buffer, pH 4·0), clotrimazole, miconazole and glibenclamide, which were dissolved in dimethylsulphoxide (DMSO), indomethacin and 17-ODYA, which were dissolved in ethanol, and levcromakalim, for which 70% ethanol was used. Except for 1-ABT, all substances were added from the appropriate stock solutions to aliquots of the equilibrated (95% O_2–5% CO_2; 35 °C) Krebs–Ringer solution a few minutes before superfusion. The final solvent concentration never exceeded 0·2% for DMSO or 0·07% for ethanol, concentrations which had no influence on the measured electrophysiological parameters. All drug concentrations are expressed as final molar concentrations in the superfusate.

Statistics

Results are expressed as means ± S.E.M. Statistical significance was evaluated using Student's t test for paired or unpaired observations, a P value $< 0·05$ indicating a significant difference; n indicates the number of preparations, each obtained from a different rat.

RESULTS

Endothelium-dependent responses

Characterization of the membrane electrical response to ACh. In mesenteric artery strips, the resting V_m was stable and averaged $-50·2 \pm 0·5$ mV ($n = 49$). Prolonged (10 min) exposure of vessel strips with endothelium to ACh (1 μM) in the superfusate produced a hyperpolarization with a characteristic time course (Chen & Suzuki, 1989; Vanheel *et al.* 1994), typical examples of which can be seen in Fig. 1. After addition of ACh, V_m hyperpolarized to a peak value of $-68·3 \pm 1·0$ mV ($n = 28$). In the prolonged presence of the agonist, the peak hyperpolarization, due to EDHF (Chen & Suzuki, 1989; Fujii *et al.* 1992), was followed by a partial recovery of V_m to a level that was somewhat more negative than the control resting V_m. After 10 min in the presence of ACh, V_m tended to stabilize at a level of $-56·9 \pm 0·8$ mV. Part of this sustained component of hyperpolarization is caused by endogenous NO liberated from the endothelium, as has been shown in the rat aorta (Vanheel *et al.* 1994) and the coronary artery (Parkington *et al.* 1993). After washout of the agonist, V_m depolarized back to its resting level.

As observed in the rat aorta (Vanheel *et al.* 1994), a progressive decrease of the ACh-induced hyperpolarization was noted with repeated administrations during the course of the experiments. This decrease can also be observed by comparing, for example, the first and the last hyperpolarization in Fig. 1*B*. The rate and extent of this 'run-

Table 1. Influence of the various inhibitors on V_m and its response to ACh

Inhibitor	Hyperpolarization			ΔV_m drug
	Control (mV)	Drug (mV)	After (mV)	(mV)
Glibenclamide (10 μM)	−15·2 ± 1·5 (5)	−11·9 ± 1·6 (4)	−8·3 ± 1·9 (5)	1·6 ± 0·8 (5)
Quinacrine (30 μM)	−17·3 ± 1·4 (4)	1·0 ± 0·6 (4)***	−7·1 ± 2·5 (3)†	1·6 ± 0·8 (4)
Indomethacin (50 μM)	−17·5 ± 1·5 (4)	−13·9 ± 1·0 (3)	−11·0 ± 0·2 (2)	−1·1 ± 0·4 (4)
Proadifen (30 μM)	−13·8 ± 2·8 (5)	−0·2 ± 0·2 (5)**	−3·3 ± 0·7 (4)†	1·9 ± 0·7 (5)
Clotrimazole (30 μM)	−17·8 ± 1·8 (6)	−10·3 ± 1·3 (6)**	−6·1 ± 3·7 (3)	1·1 ± 0·3 (7)‡
Miconazole (100 μM)	−23·0 ± 0·8 (5)	−8·9 ± 3·3 (7)**	−22·7 ± 0·9 (3)†	5·7 ± 0·6 (7)‡‡‡
17-ODYA (5 μM)	−18·2 ± 1·3 (5)	−14·9 ± 1·8 (5)	−13·0 ± 1·9 (5)	−0·6 ± 0·3 (4)
1-ABT (2 mM)	−16·7 ± 1·9 (4)	−14·1 ± 2·0 (4)	−13·2 ± 0·2 (3)	−2·6 ± 0·3 (5)‡‡

Magnitude of the ACh-induced endothelium-dependent hyperpolarization before (Control), in the presence (Drug) and after washout (After) of the different inhibitors. The influence of the drugs on the resting membrane potential is also given (ΔV_m drug). Values are means ± S.E.M.; the number of experiments is given in parentheses. ** $P < 0.01$, *** $P < 0.001$, significantly different from the ACh-induced hyperpolarization under control conditions (Control); † $P < 0.05$, significantly different from the hyperpolarization in the presence of the drug (Drug); ‡ $P < 0.05$, ‡‡ $P < 0.01$, ‡‡‡ $P < 0.001$, significantly different change.

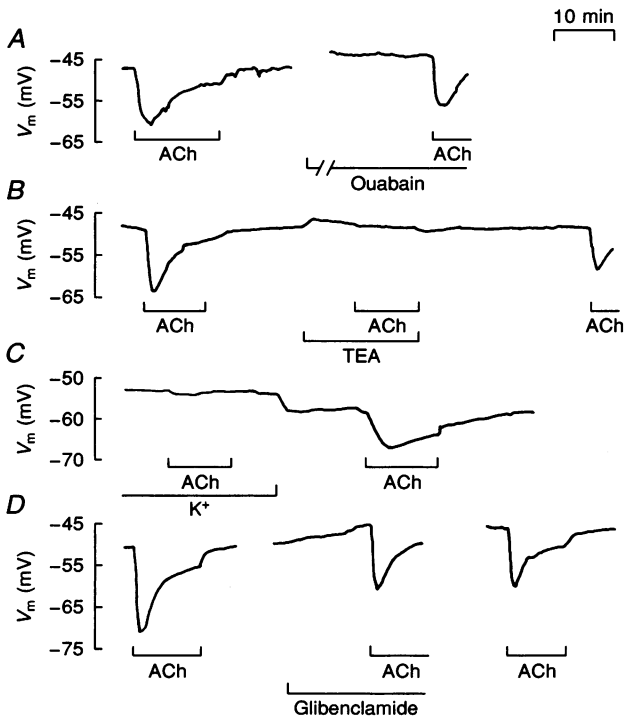

Figure 1. ACh-induced endothelium-dependent hyperpolarization is caused by opening of K^+ channels

Original traces of membrane potential (V_m) responses to 10 min applications of ACh (1 μM) in smooth muscle cells from the mesenteric artery. *A–D* show traces from 4 different preparations. Endothelium-dependent hyperpolarizations consist of an initial peak followed by a partial recovery of V_m. *A*, 10 μM ouabain does not inhibit endothelium-dependent hyperpolarization. Superfusion with 3 mM tetraethylammonium (TEA; *B*) and by high K^+ (15·6 mM)-containing solution (*C*) reversibly inhibits the electrical response. Glibenclamide (10 μM) does not significantly influence the response to ACh (*D*).

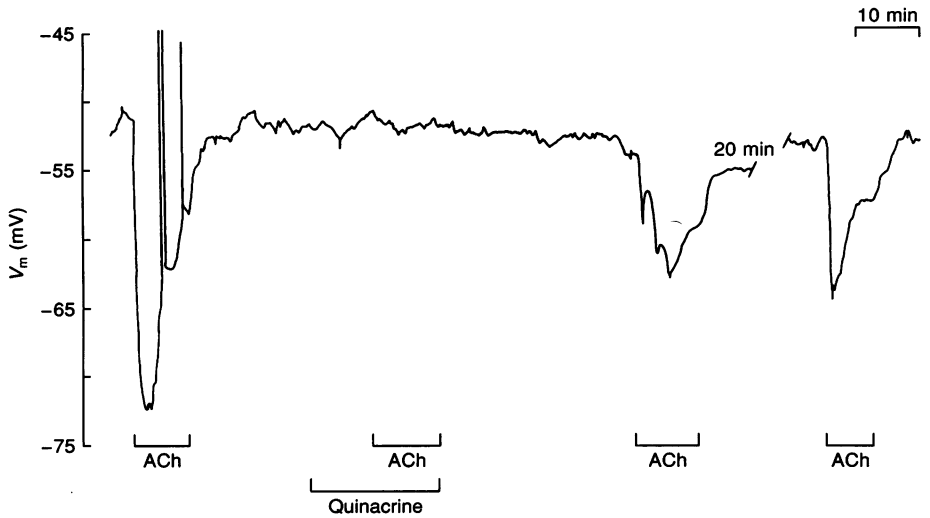

Figure 2. Phospholipase A_2 inhibition reversibly blocks endothelium-dependent hyperpolarization

V_m responses to ACh (1 μM) before, during and after application of quinacrine (30 μM). Vertical lines during the first ACh exposure are due to microelectrode dislodgement and reimpalement. During the break in the record the preparation was superfused for 20 min with control fluid without microelectrode displacement from the cell.

down' was variable between different preparations, the membrane response to a second application of ACh being reduced by 5–20%.

Pre-exposure of the vessel strips to ouabain (10 μM) did not have a significant effect on the peak hyperpolarization induced by ACh (Fig. 1*A*). Exposure to 3 mM TEA depolarized V_m by 2–3 mV. In the presence of this rather non-specific K^+ channel inhibitor, the membrane hyperpolarization produced by ACh was virtually completely and fully reversibly inhibited (Fig. 1*B*). Superfusion with 15·6 mM K^+-containing solution drastically diminished the hyperpolarization to ACh (Fig. 1*C*). The specific inhibitor of K_{ATP} channels, glibenclamide, at a concentration of 10 μM, failed to significantly influence the membrane electrical change in response to ACh (Fig. 1*D*). The mean values of the ACh-induced hyperpolarization before, in the presence of, and after washout of the drug are listed in Table 1.

Influence of quinacrine on endothelium-dependent responses. Quinacrine (30 μM) did not have a significant effect on the resting V_m. In the presence of the substance, the hyperpolarization in response to ACh was completely inhibited (Table 1). This influence was only partially reversible. A typical experiment in which the microelectrode impalement was kept throughout the exposure to and washout of quinacrine is depicted in Fig. 2. After washout of the substance, the endothelium-dependent V_m change

Figure 3. Cyclo-oxygenase inhibition does not impair ACh-induced hyperpolarization

The endothelium-dependent V_m responses to ACh (1 μM) were measured in the absence and presence of indomethacin (50 μM).

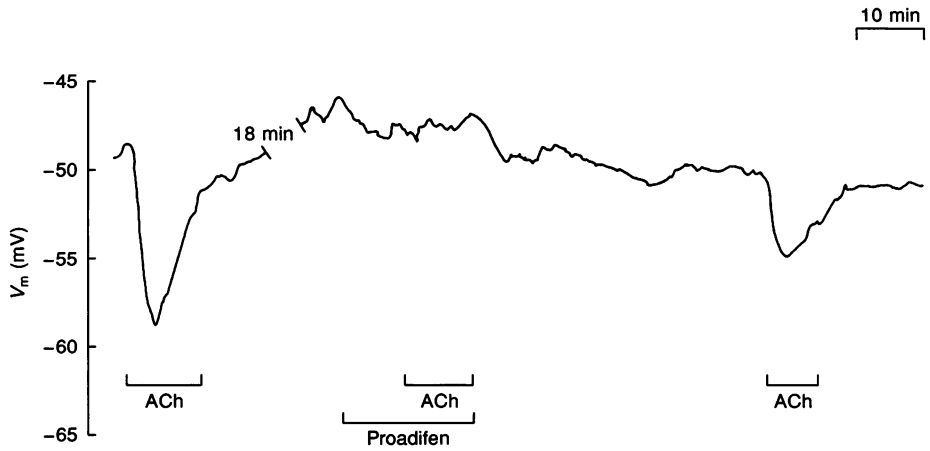

Figure 4. Proadifen reversibly inhibits endothelium-dependent hyperpolarization
V_m responses to ACh (1 μM) before, during and after application of 30 μM proadifen. During the break in the record, the preparation was superfused for 18 min with the control fluid.

produced by ACh reappeared. In the experiment shown in Fig. 2, the hyperpolarization during the first ACh challenge after quinacrine removal occurred hesitatingly but later on the V_m change regained its regular time course.

Influence of indomethacin. The hyperpolarization produced by ACh (1 μM) was not significantly affected by pre-exposure of the preparation to a high concentration (50 μM) of the inhibitor of the cyclo-oxygenase pathway of arachidonic acid metabolism (Fig. 3 and Table 1).

Influence of cytochrome P450 inhibitors. The application of 30 μM proadifen virtually completely inhibited the membrane hyperpolarization to 1 μM ACh (Fig. 4 and Table 1). After washout of the drug, the V_m response to the agonist reappeared (Fig. 4).

The imidazole antimycotic clotrimazole (30 μM) slightly depolarized the resting V_m (Fig. 5*A* and Table 1). In the presence of the cytochrome P450 inhibitor, the peak hyperpolarization produced by ACh was decreased to

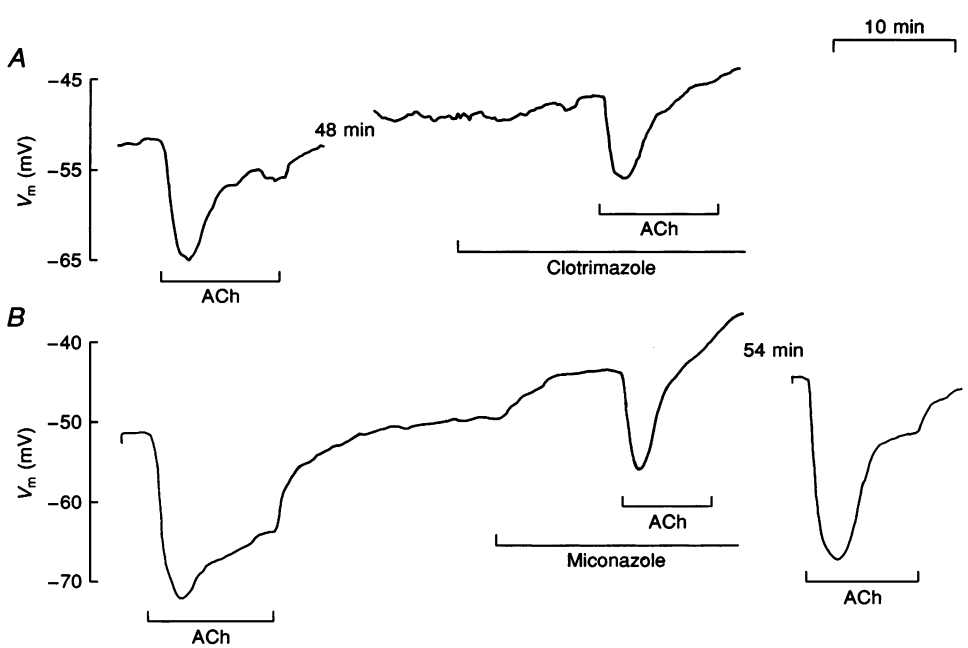

Figure 5. Effect of imidazole antimycotics on endothelium-dependent responses
Endothelium-dependent hyperpolarizations of V_m induced by ACh (1 μM) were measured in the absence and presence of 30 μM clotrimazole (*A*) and 100 μM miconazole (*B*). During the break in the records the preparation was superfused for the indicated time with the control solution.

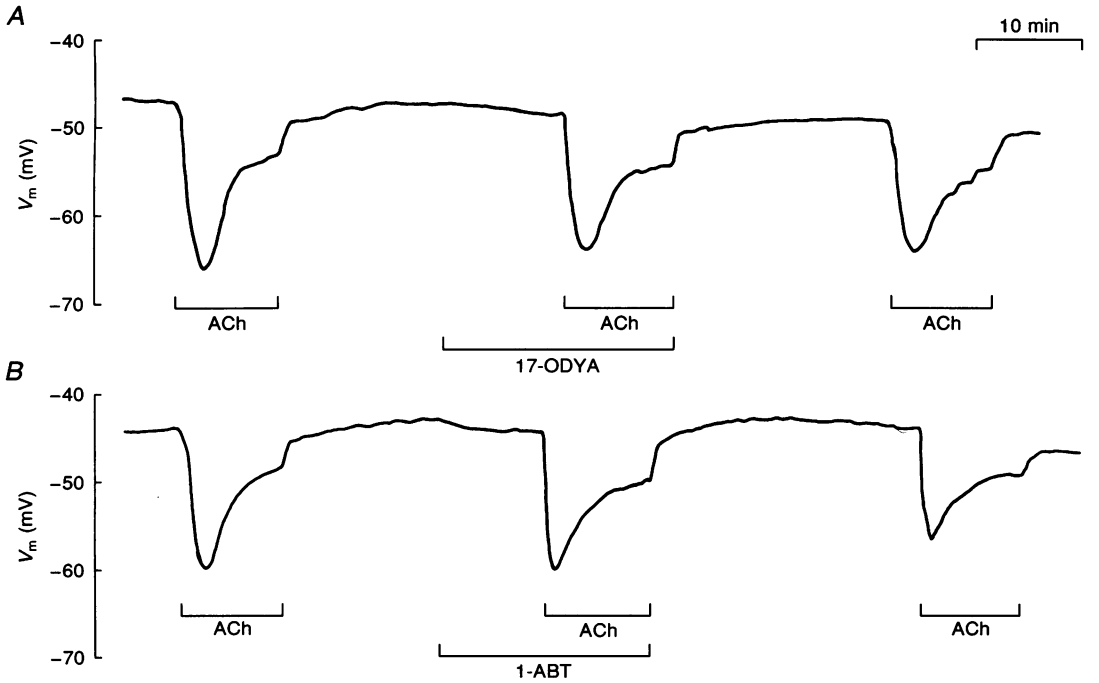

Figure 6. Lack of influence of the cytochrome P450 inhibitors 17-ODYA and 1-ABT

V_m recordings in the rat main mesenteric artery. Traces from 2 different experiments, in which the influence of pre-exposure to 17-ODYA (5 μM; *A*) and 1-ABT (2 mM; *B*) on the endothelium-dependent hyperpolarization induced by ACh (1 μM) was tested.

10·3 ± 1·3 mV compared with 17·8 ± 1·8 mV under control conditions in this group of preparations. A further, more marked and consistent observation was a large inhibitory effect on the sustained V_m change in the continuous presence of ACh. Whereas under control conditions V_m in the presence of ACh remained significantly hyperpolarized with respect to the resting level, in the presence of clotrimazole it was not significantly different from the resting V_m in the absence of the mediator (Fig. 5*A*). Following washout of clotrimazole, the peak hyperpolarization averaged 6·1 ± 3·7 mV ($n = 3$).

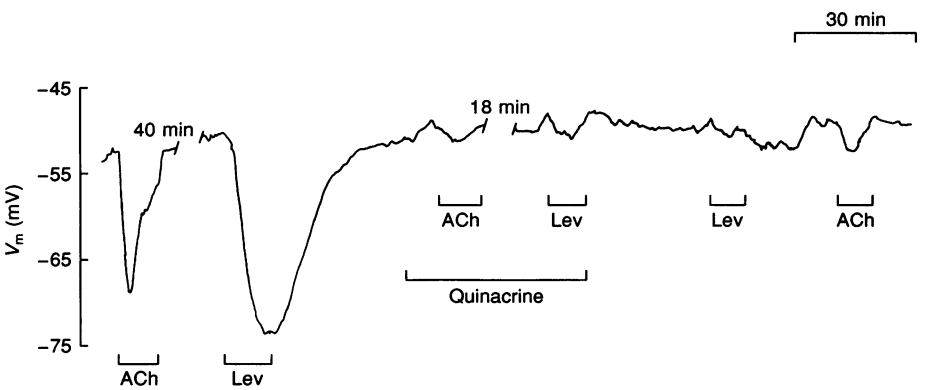

Figure 7. Quinacrine inhibits endothelium-independent hyperpolarization induced by K_{ATP} channel opening

V_m changes of mesenteric artery smooth muscle cells in response to 300 nM levcromakalim (Lev) before, during and after exposure to quinacrine (30 μM). For comparison, the influence of quinacrine on ACh (1 μM)-induced response was also tested in this preparation. During the breaks in the record, the preparation was superfused for the indicated time with, respectively, control solution and quinacrine-containing superfusate.

Miconazole (100 μM) significantly depolarized the resting V_m by about 6 mV (Fig. 5*B* and Table 1). After 10 min pre-exposure to this cytochrome P450 inhibitor, the peak hyperpolarization in response to ACh was significantly decreased to 8·9 ± 3·3 mV ($n = 7$). As was observed with clotrimazole, the ACh-induced hyperpolarization was significantly abbreviated, the sustained component being totally inhibited (Fig. 5*B*). This inhibitory effect was partially reversible (Fig. 5*B*).

After pre-exposure to 5 μM 17-ODYA, the ACh-induced hyperpolarization was not significantly affected (Fig. 6*A* and Table 1). Similarly, 1-ABT at a concentration of 2 mM, which hyperpolarized the resting V_m by 2·6 mV, did not have a significant effect on the ACh-induced hyperpolarization (Fig. 6*B* and Table 1).

Endothelium-independent responses

Levcromakalim, a selective opener of K_{ATP} channels, hyperpolarized V_m in a concentration-dependent manner (see e.g. Fig. 9*A*). In order to induce a hyperpolarization similar to that obtained with 1 μM ACh, we usually applied 300 nM levcromakalim, producing a mean V_m change of −15·6 ± 0·9 mV ($n = 21$) under control conditions. This hyperpolarization was endothelium independent and was completely prevented by pre-exposure to 10 μM glibenclamide (not shown).

Influence of quinacrine. Quinacrine (30 μM) nearly completely prevented the hyperpolarization produced by 300 nM levcromakalim (mean V_m change, −1·3 ± 0·8 mV; $n = 3$; $P < 0·001$). A representative experiment in which exposures of a muscle strip to ACh and levcromakalim were alternated in the absence, presence and after washout of quinacrine is shown in Fig. 7. The inhibitory influence of quinacrine on the hyperpolarization produced by levcromakalim was less reversible than on that produced by ACh. After washout of quinacrine, the V_m change produced by 300 nM levcromakalim was −0·7 ± 0·7 mV ($n = 3$).

Influence of cytochrome P450 inhibitors. Pre-exposure to proadifen completely inhibited the hyperpolarization produced by 300 nM levcromakalim ($n = 3$; $P < 0·001$). This influence was only partially reversible within the time limits of the experiments (Fig. 8).

Clotrimazole (30 μM) completely blocked the membrane hyperpolarization produced by 300 nM levcromakalim ($n = 3$; $P < 0·001$), although higher concentrations of the K^+ channel opener could still elicit small changes in V_m (Fig. 9*A*). In two experiments, the influence of levcromakalim could also be tested after washout of clotrimazole. Both showed irreversible inhibition (see Fig. 9*A*).

Miconazole (100 μM) also inhibited the peak hyperpolarization by 300 nM levcromakalim ($n = 4$; $P < 0·001$). Moreover, the inhibition was irreversible, while the peak hyperpolarization in response to ACh was little affected (Fig. 9*B*) following washout of the cytochrome P450 inhibitor.

Neither 17-ODYA (5 μM) nor 1-ABT (2 mM) significantly influenced the levcromakalim-induced hyperpolarization (Fig. 10; $n = 3$, $P = 0·4$ for 17-ODYA; and $n = 2$ for 1-ABT).

DISCUSSION

The present studies confirm previous findings in various vessels. ACh produces a hyperpolarization of the V_m which is endothelium-dependent and displays, in the rat main mesenteric artery (Fukao, Hattori, Kanno, Sakuma & Kitabatake, 1995), as well as in the rat aorta (Chen &

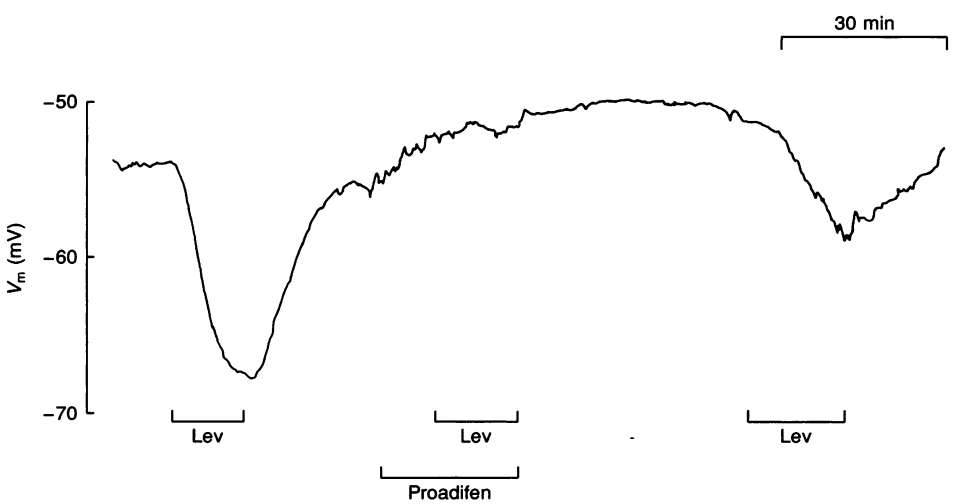

Figure 8. Proadifen inhibits endothelium-independent hyperpolarization induced by K_{ATP} channel opening

V_m changes of mesenteric artery smooth muscle cell induced by 300 nM levcromakalim (Lev) before, during and after exposure to proadifen (30 μM).

Suzuki, 1989; Vanheel *et al.* 1994), a rather characteristic time course. An initial transient peak hyperpolarization is followed by a smaller and more sustained component of hyperpolarization (Chen & Suzuki, 1989). The latter phase is partly due to endogenous NO liberated from the endothelium, as has been shown in the rat aorta (Vanheel *et al.* 1994) and the coronary artery (Parkington *et al.* 1993). Exogenous NO indeed slightly (~2 mV) hyperpolarizes the main mesenteric artery under our experimental conditions (Vanheel & Van de Voorde, 1995). The transient peak hyperpolarization is mainly caused by release of EDHF. It occurs by activation of smooth muscle K^+ channels since: (a) ouabain, which blocks the Na^+ pump, has no significant effect on this hyperpolarization; (b) decreasing the outwardly directed driving force for K^+ ions greatly reduces the hyperpolarization; and (c) the rather non-specific K^+ channel inhibitor TEA reversibly blocks the peak V_m change (Fig. 1). As reported for several rat vessels including mesenteric arteries (McPherson & Angus, 1991; Fujii *et al.* 1992), glibenclamide does not have a large effect on the ACh-induced endothelium-dependent hyperpolarization (Fig. 1*D* and Table 1), suggesting that the K^+ channels activated by EDHF are different from those activated by levcromakalim. Since glibenclamide is known to block the (slower) hyperpolarization induced by iloprost (Murphy & Brayden, 1995), the limited and statistically insignificant influence of the sulphonylurea on the ACh-induced peak hyperpolarization further confirms the nominal absence of a prostacyclin-mediated component in this rapid phase of hyperpolarization in rat tissue (Parkington *et al.* 1993), which is predominantly due to EDHF.

Several studies in which the nitro-L-arginine-resistant, thus NO-independent, component of vasodilatation was measured suggested that the hyperpolarizing factor might be (a) cytochrome P450 mono-oxygenase-generated metabolite(s) from arachidonic acid (Hecker *et al.* 1994; Bauersachs *et al.* 1994; Fulton, Mahboubi, McGiff & Quilley, 1995). In coronary arteries, the biosynthesis of several EETs has been shown (Rosolowski, Falck, Willerson & Campbell, 1990). In addition, all EET regioisomers have been shown to activate K_{Ca} channels in isolated smooth muscle cells from several arteries (Gebremedhin *et al.* 1992; Hu & Kim, 1993; Campbell *et al.* 1996) and are, therefore, possible candidates as EDHF.

In the present experiments on rat mesenteric arteries, we found that quinacrine reversibly inhibited the EDHF-

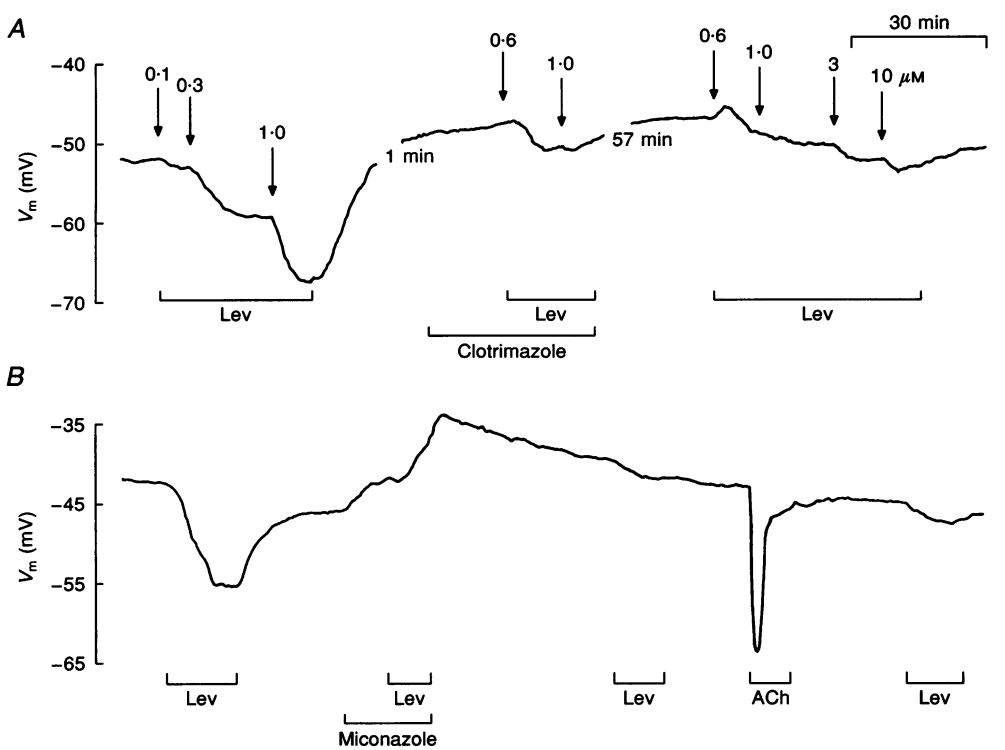

Figure 9. Imidazole antimycotics inhibit endothelium-independent hyperpolarization induced by K_{ATP} channel opening

Original tracings of two long-term V_m measurements in mesenteric artery cells in which the influence of 30 μM clotrimazole (*A*) and 100 μM miconazole (*B*) on the hyperpolarization induced by levcromakalim (Lev) was tested. In *A*, cumulative concentrations (μM) of the K^+ channel opener were applied as indicated by the arrows. During the breaks in the trace, the preparation was superfused for the indicated time with the control fluid. In *B*, a fixed levcromakalim concentration (300 nM) was used to elicit hyperpolarization. Note inhibition of hyperpolarization by miconazole and intact peak hyperpolarization to ACh (1 μM) after washout of miconazole, while responses to levcromakalim were still inhibited.

induced peak hyperpolarization elicited by ACh. In addition, proadifen, which is metabolized to an intermediate that inhibits cytochrome P450, fully blocked the ACh-induced hyperpolarization. These findings are consistent with the hypothesis that the endothelium-dependent hyperpolarizing factor is a cytochrome P450-derived metabolite from arachidonic acid (Hecker *et al.* 1994; Fulton *et al.* 1995). However, from these experiments an inhibitory influence of both quinacrine and proadifen on the liberation of this factor and/or on the activation of the muscular K^+ channels by this substance cannot be excluded. Both drugs, for example, have been shown to possess calmodulin-antagonizing activity (Volpi, Sharafi, Epstein, Andrenyak & Feinstein, 1981), and calmodulin antagonists have been shown to inhibit the endothelium-dependent hyperpolarization (Nagao, Iliano & Vanhoutte, 1992). In the present study, both compounds inhibited the endothelium-independent hyperpolarization produced by the K^+ channel opener levcromakalim (Figs 7 and 8). This directly indicates that the activation of at least the K_{ATP} channels in the membrane of the vascular smooth muscle cells is inhibited by these drugs.

The antifungal agents clotrimazole and miconazole inhibit cytochrome P450 by the formation of nitrogenous ligands to the haem iron of the protein. In the present experiments, we found that 10 min pre-exposure to 30 μM clotrimazole or 100 μM miconazole, concentrations considerably higher than their reported IC_{50} values, totally inhibited the levcromakalim-induced hyperpolarization but had less effect on the peak endothelium-dependent hyperpolarization produced by ACh (Fig. 5 and Table 1). In addition, we showed that two other structurally different inhibitors of cytochrome P450, 17-ODYA, which specifically blocks cytochrome P450-dependent mono-oxygenase-mediated long chain fatty acid metabolism, and 1-ABT, which is oxidized to benzyne which alkylates the porphyrin structure of the haemoprotein, had no influence on the ACh-induced hyperpolarization. These findings are a strong argument against the proposal that the endothelium-dependent hyperpolarizing factor is a cytochrome P450-derived metabolite from arachidonic acid, at least in the rat mesenteric artery. Similar findings were reported recently for the rat hepatic and the guinea-pig carotid artery (Zygmunt, Edwards, Weston, Davis & Högestätt, 1996; Corriu, Félétou, Canet & Vanhoutte, 1996). In the rat vessel, proadifen decreased the ACh-induced hyperpolarization from 14 to 3 mV, but completely abolished the hyperpolarization (23–25 mV) by levcromakalim. In addition, the more specific inhibitor of cytochrome P450 mono-oxygenases, 17-ODYA, failed to inhibit the EDHF-mediated endothelium-dependent relaxation (Zygmunt *et al.* 1996).

Imidazole antimycotic agents have been reported to inhibit Ca^{2+}-dependent K^+ channels (Alvarez, Montero & Garcia-Sancho, 1992) as well as voltage-dependent K^+ channels (Yuan, Tod, Rubin & Blaustein, 1995). Both proadifen

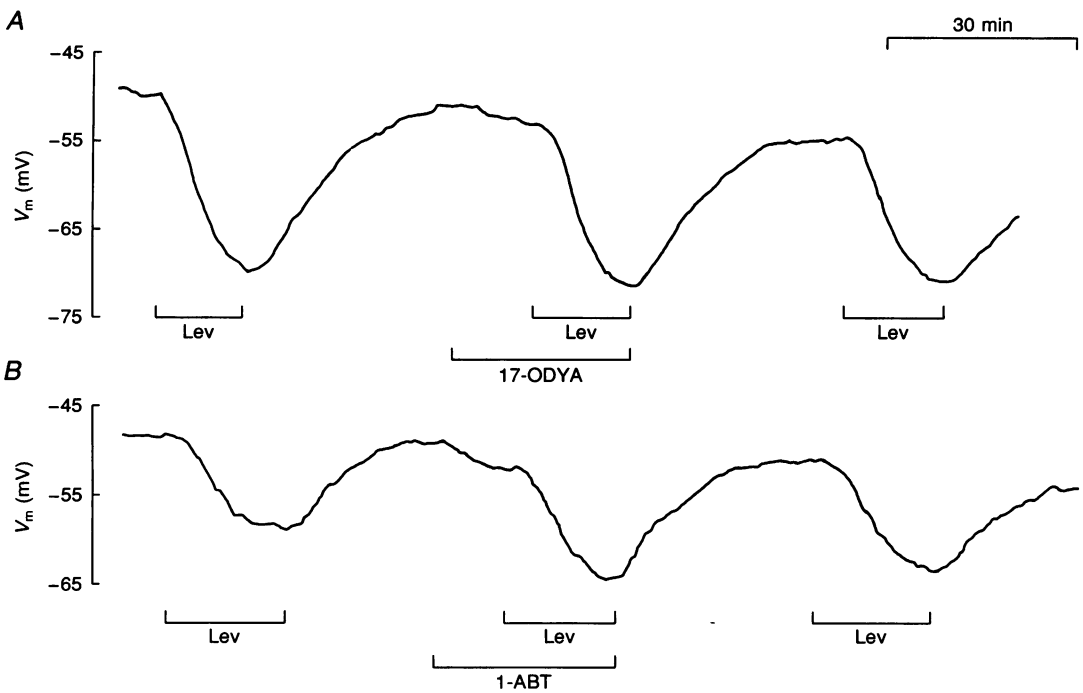

Figure 10. 17-ODYA or 1-ABT do not impair hyperpolarization induced by K_{ATP} channel opening

Traces from 2 different experiments in which the influence of 17-ODYA (5 μM; *A*) and 1-ABT (2 mM; *B*) on the endothelium-independent hyperpolarization elicited by levcromakalim (Lev; 300 nM) was tested.

(10 μM) and clotrimazole (30 μM) have recently been reported to inhibit the delayed rectifier and the ATP-regulated K^+ current ($I_{K(ATP)}$) in freshly isolated rat portal vein smooth muscle cells, while 17-ODYA (5 μM) had no effect on $I_{K(ATP)}$ (Edwards, Zygmunt, Högestätt & Weston, 1996). In the present study, we found that both imidazole antimycotics completely and nearly irreversibly inhibited the hyperpolarization produced by levcromakalim, while they had much less influence on the peak endothelium-dependent hyperpolarization produced by ACh (compare Figs 9 and 5). This indicates that K_{ATP} channels are more sensitive to these agents than the channels activated by EDHF, and adds further evidence that in smooth muscle cells from the rat main mesenteric artery the action of EDHF is not mediated by activation of K_{ATP} channels.

The influence of clotrimazole and miconazole on the later component of the ACh-induced hyperpolarization is similar to the effect of inhibition of NO synthase in the rat aorta (Vanheel *et al.* 1994) and might possibly be explained by partial inhibition of the release and/or of the hyperpolarizing influence of NO. The NO synthase enzyme contains a haem centre with the spectral properties of cytochrome P450, and shows considerable sequence homology with the NADPH-dependent cytochrome P450 reductase (for review, see Knowles & Moncada, 1994). Cytochrome P450 inhibitors, especially those interacting with the haem group, might therefore influence NO synthase activity. In fact, early studies suggested that the endothelium-dependent relaxing factor, later identified as NO, was a lipoxygenase or cytochrome P450 metabolite of arachidonic acid (Singer & Peach, 1983). In bovine aortic endothelial cells, miconazole has been shown to directly inhibit the constitutive NO synthase (Dudek, Conforto, Pinto, Wildhirt & Suzuki, 1995). Furthermore, the hyperpolarizing effect of NO is known to be mediated by K_{ATP} channels, at least in small mesenteric arteries from the rat (Garland & McPherson, 1992). If endogenous NO liberated by ACh would act on the same type of K^+ channel in the main mesenteric artery, one might expect that the imidazole antimycotics, documented in the present study to inhibit K_{ATP} channel opening, were more effective inhibitors of the later component of hyperpolarization (induced by NO) than on the peak endothelium-dependent hyperpolarization (induced by EDHF). The observed depolarization of the resting V_m by miconazole is also in line with inhibition of basal NO release.

In conclusion, it was found that mechanistically dissimilar inhibitors of cytochrome P450 mono-oxygenase have a dissimilar effect on the endothelium-dependent hyperpolarization elicited by ACh in the rat mesenteric artery. Proadifen, which inhibited the endothelium-dependent hyperpolarization, also inhibited the endothelium-independent hyperpolarization induced by levcromakalim, showing an inhibitory effect on the target for EDHF, the vascular smooth muscle cell. Inhibition of the K^+ channels activated by EDHF, therefore, is just as likely to explain the blocked endothelium-dependent hyperpolarization (and NO-independent relaxation) as inhibition of the formation of EDHF. Imidazole antimycotics irreversibly inhibited the levcromakalim-induced hyperpolarization, but had much less effect on the (peak) endothelium-dependent hyperpolarization induced by ACh. Moreover, two different suicide substrate inhibitors of cytochrome P450-dependent arachidonic acid metabolism, 17-ODYA and 1-ABT, had no influence on EDHF-mediated hyperpolarization. Taken together, these findings do not support the view that the ACh-induced endothelium-dependent hyperpolarization in the rat mesenteric artery is caused by (a) cytochrome P450 mono-oxygenase-derived metabolite(s) of arachidonic acid.

Alvarez, J., Montero, M. & Garcia-Sancho, J. (1992). High affinity inhibition of Ca^{2+}-dependent K^+ channels by cytochrome P-450 inhibitors. *Journal of Biological Chemistry* **267**, 11789–11793.

Bauersachs, J., Hecker, M. & Busse, R. (1994). Display of the characteristics of endothelium-derived hyperpolarizing factor by a cytochrome P450-derived arachidonic acid metabolite in the coronary microcirculation. *British Journal of Pharmacology* **113**, 1548–1553.

Campbell, W. G., Gebremedhin, D., Pratt, P. F. & Harder, D. R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circulation Research* **78**, 415–423.

Chen, G. & Suzuki, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *Journal of Physiology* **410**, 91–106.

Chen, G., Suzuki, H. & Weston, A. H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *British Journal of Pharmacology* **95**, 1165–1174.

Corriu, C., Félétou, M., Canet, E. & Vanhoutte, P. M. (1996). Inhibitors of the cytochrome P450-mono-oxygenase and endothelium-dependent hyperpolarization in the guinea-pig isolated coronary artery. *British Journal of Pharmacology* **117**, 607–610.

Dudek, R. R., Conforto, A., Pinto, V., Wildhirt, S. & Suzuki, H. (1995). Inhibition of endothelial nitric oxide synthase by cytochrome P-450 reductase inhibitors. *Proceedings of the Society for Experimental Biology and Medicine* **209**, 60–64.

Edwards, G., Zygmunt, P. M., Högestätt, E. D. & Weston, A. H. (1996). Effects of cytochrome P450 inhibitors on potassium currents and mechanical activity in rat portal vein. *British Journal of Pharmacology* **119**, 691–701.

Félétou, M. & Vanhoutte, P. M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *British Journal of Pharmacology* **93**, 515–524.

Fujii, K., Tominaga, M., Ohmori, S., Kobayashi, K., Koga, T., Takata, Y. & Fujishima, M. (1992). Decreased endothelium dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. *Circulation Research* **70**, 660–669.

Fukao, M., Hattori, Y., Kanno, M., Sakuma, I. & Kitabatake, A. (1995). Thapsigargin- and cyclopiazonic acid-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *British Journal of Pharmacology* **115**, 987–992.

Fulton, D., Mahboubi, K., McGiff, J. C. & Quilley, J. (1995). Cytochrome P450-dependent effects of bradykinin in the rat heart. *British Journal of Pharmacology* **114**, 99–102.

FURCHGOTT, R. F. & VANHOUTTE, P. M. (1989). Endothelium-derived relaxing and constricting factors. *FASEB Journal* **3**, 2007–2018.

GARLAND, C. J. & MCPHERSON, G. A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *British Journal of Pharmacology* **105**, 429–435.

GEBREMEDHIN, D., MA, Y. H., FALCK, J. R., ROMAN, R. J., VANROLLINS, M. & HARDER, D. R. (1992). Mechanism of action of cerebral epoxyeicosatrienoic acids in cerebral arterial smooth muscle. *American Journal of Physiology* **263**, H519–525.

HECKER, M., BARA, A., BAUERSACHS, J. & BUSSE, R. (1994). Characterization of endothelium-dependent hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *Journal of Physiology* **481**, 407–414.

HU, S. & KIM, H. S. (1993). Activation of K^+ channels in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. *European Journal of Pharmacology* **230**, 215–221.

IGNARRO, L. J. & KADOWITZ, P. J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annual Review of Pharmacology and Toxicology* **25**, 171–191.

KNOWLES, R. G. & MONCADA, S. (1994). Nitric oxide synthases in mammals. *Biochemical Journal* **298**, 249–258.

MCPHERSON, G. A. & ANGUS, J. A. (1991). Evidence that acetylcholine-mediated hyperpolarization of the rat small mesenteric artery does not involve the K^+ channel opened by cromakalim. *British Journal of Pharmacology* **103**, 1184–1190.

MURPHY, M. E. & BRAYDEN, J. E. (1995). Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *Journal of Physiology* **486**, 47–58.

NAGAO, T., ILIANO, S. & VANHOUTTE, P. M. (1992). Calmodulin antagonists inhibit endothelium-dependent hyperpolarization in the canine coronary artery. *British Journal of Pharmacology* **107**, 382–386.

PARKINGTON, H. C., TARE, M., TONTA, M. A. & COLEMAN, H. A. (1993). Stretch revealed three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. *Journal of Physiology* **465**, 459–476.

RAPOPORT, R. M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. *Circulation Research* **52**, 352–357.

REES, D. D., PALMER, R. M. J., SCHULTZ, R., HODSON, H. F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *British Journal of Pharmacology* **101**, 746–752.

ROSOLOWSKI, M., FALCK, J. A., WILLERSON, J. T. & CAMPBELL, W. B. (1990). Synthesis of lipoxygenase and epoxygenase products of arachidonic acid by normal and stenosed canine coronary arteries. *Circulation Research* **66**, 608–621.

SINGER, H. A. & PEACH, M. J. (1983). Endothelium-dependent relaxation of rabbit aorta. II. Inhibition of relaxation stimulated by metacholine and A23187 with antagonists of arachidonic acid metabolism. *Journal of Pharmacology and Experimental Therapeutics* **226**, 796–801.

TARE, M., PARKINGTON, H. C., COLEMAN, H. A., NEILD, T. O. & DUSTING, G. J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature* **346**, 69–71.

TAYLOR, S. G. & WESTON, A. H. (1988). Endothelium-derived hyperpolarizing factor: A new endogenous inhibitor from the vascular endothelium. *Trends in Pharmacological Sciences* **9**, 272–274.

VAN DE VOORDE, J., VANHEEL, B. & LEUSEN, I. (1992). Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. *Circulation Research* **70**, 1–8.

VANHEEL, B. & VAN DE VOORDE, J. (1995). Membrane potential responses to endothelium-dependent and -independent vasodilators in rat aorta and mesenteric arteries. *Pflügers Archiv* **430**, R140 (abstract).

VANHEEL, B., VAN DE VOORDE, J. & LEUSEN, I. (1994). Contribution of nitric oxide to the endothelium-dependent hyperpolarization in rat aorta. *Journal of Physiology* **475**, 277–284.

VOLPI, M., SHARAFI, P. M., EPSTEIN, P. M., ANDRENYAK, D. M. & FEINSTEIN, M. B. (1981). Local anesthetics, mepacrine and propanolol are antagonists of calmodulin. *Proceedings of the National Academy of Sciences of the USA* **78**, 795–801.

YUAN, X.-J., TOD, M. L., RUBIN, L. J. & BLAUSTEIN, M. P. (1995). Inhibition of cytochrome P-450 reduces voltage-gated K^+ currents in pulmonary arterial myocytes. *American Journal of Physiology* **268**, C259–270.

ZYGMUNT, P. M., EDWARDS, G., WESTON, A. H., DAVIS, S. C. & HÖGESTÄTT, E. D. (1996). Effects of cytochrome P450 inhibitors on EDHF-mediated relaxation in the rat hepatic artery. *British Journal of Pharmacology* **118**, 1147–1152.

ZYGMUNT, P. M. & HÖGESTÄTT, E. D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitro-arginine in the rat hepatic artery. *British Journal of Pharmacology* **117**, 1600–1606.

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