Components of acetylcholine-induced dilation in isolated rat arterioles

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Bakker, Erik N. T. P., and Pieter Sipkema. Components of acetylcholine-induced dilation in isolated rat arterioles. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1848–H1853, 1997.—Acetylcholine-induced dilation was studied in cannulated resistance arteries of rat cremaster muscle. Pressurized arteriolar segments (internal diameter: 175 ± 2 µm) developed spontaneous tone (90 ± 2% of 4.0 µM acetylcholine (0.1 and 0.3 µM) resulted in a transient dilation followed by a steady-state dilatory response. In the presence of Nω-nitro-L-arginine (L-NNa) ~70% of the transient dilation was resistant to nitric oxide inhibition, whereas the steady-state response was abolished. Further experiments using 0.1 µM acetylcholine (no L-NNa present) were aimed to inhibit the transient component of the response. 4.5 µM acetylcholine (no L-NNa present) inhibited the transient component of the response (1.3 ± 1.3% of 4.5 pM acetylcholine, and SKF-525a (4.8 ± 4.5 µM) abolished this transient dilation (0.3% ± 1.3% of 4.5 pM acetylcholine, suggesting that the dilation is mediated by an endothelium-derived hyperpolarizing factor (EDHF). This putative EDHF-mediated dilation is strongly reduced by cytochrome P-450 inhibitors miconazole (11 ± 1.3% of 4.5 pM acetylcholine) and SKF-525a (4.8 ± 4.5 µM). The transient component is inhibited by tetraethylammonium nitrate but not by glibenclamide, indicating it is mediated by a potassium ion selectivity filter (10.5 ± 1.3% of 4.5 pM acetylcholine). Interestingly, inhibition of the transient component was followed by a subsequent increase of potassium ion selectivity filter of the response to acetylcholine. Thus a transient dilation, mediated by a cytochrome P-450 metabolite, precedes and possibly stimulates nitric oxide-mediated dilation in acetylcholine-induced dilation.

Nitric oxide; endothelium-derived hyperpolarizing factor; cytochrome P-450; resistance vessels; potassium channels

ENDOTHELIUM-DEPENDENT dilation induced by agonists like acetylcholine is partially resistant to inhibitors of nitric oxide-mediated dilation (15, 18, 22). Acetylcholine also evokes endothelium-dependent smooth muscle hyperpolarization, which is not inhibited by L-arginine analogues, methylene blue or hemoglobin (8, 12, 19). Furthermore, exogenous nitric oxide has little affect on smooth muscle membrane potential (19, 28). Therefore, the hyperpolarizing effect of agonists like acetylcholine has been attributed to an endothelium-derived hyperpolarizing factor (EDHF). With the use of a sandwich preparation, it has been shown to be a substance transferable from an endothelium intact to an endothelium-denuded arterial segment (9). Because EDHF-mediated dilation is not affected by cyclooxygenase inhibitors, it differs from prostaglandins (8, 9, 11, 12). Another possible candidate is calcitonin gene-related peptide (CGRP). Several characteristics of CGRP-induced dilation resemble the effect of EDHF (3). CGRP is present in nerve fibers innervating the cardiovascular system and induces hyperpolarization and dilation of arteries and veins. Furthermore, the release of CGRP is enhanced by cholinergic stimulation. CGRP requires the presence of the endothelium, but it may also directly hyperpolarize smooth muscle in some of the vascular beds studied (3).

Recently, a hyperpolarizing factor induced by methacholine and bradykinin in bovine and porcine coronary arteries has been identified as a cytochrome P-450-derived arachidonic acid metabolite (5, 14). Similar findings have been reported for acetylcholine-induced EDHF release in the main mesenteric artery of the rat (6, 7). It is inhibited by high extracellular potassium concentation ([K+]o) and by two inhibitors of the Ca2+-activated K+ channel, tetraethylammonium (TEA) and charybdotoxin. Thus EDHF may function as a Ca2+-activated K+ channel opener. In support of this, cytochrome P-450 products of the epoxyeicosatrienoic acids (EETs) family were shown to open these channels using patch-clamp techniques (5).

Little is known about the physiological role of EDHF. An autocrine effect on the endothelial cell modulating the release of autacoids like nitric oxide and prostaglandins has been suggested (10). The relative importance of agonist-induced dilation that is resistant to nitric oxide inhibitors appears to be more pronounced in smaller arteries (15, 22). Therefore, the aim of our study was to investigate the contribution and nature of EDHF in acetylcholine-induced dilation in an isolated resistance arteriole. Arteriolar diameter was recorded continuously to study timing of EDHF-induced dilation. Furthermore, the effect of inhibition of EDHF on nitric oxide-mediated dilation was investigated.

MATERIALS AND METHODS

Preparation and setup. Procedures were approved by the local ethics committee for animal experimentation. Male Wistar rats of 266 ± 4 g (n = 36) were anesthetized with pentobarbital sodium (50 mg/kg ip). The right cremaster muscle was then exposed by a ventral incision of the scrotal sac and cleared from connective tissue. The muscle was opened by an incision after which the testis was removed. The cremaster was then excised and pinned to a dissecting dish containing 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (for composition see Chemicals) at 5°C. A first-order arteriole was dissected from the surrounding muscle and a 1- to 2-mm-long segment was transferred to a pressure myograph. The myograph consisted of a vessel chamber and a video camera mounted on a microscope connected to an electronic measurement system to monitor inner arteriolar diameters continuously. The vessel chamber contained two glass cannulas (tip OD ~ 100 µm), a circular heating coil, and a thermistor. The vessel chamber was filled with Krebs buffer (see below) and sealed with a glass cover. The vessel segment was mounted on one cannula and secured with a single strand of a 20-µm suture. Then, by
raising the perfusion pressure to 5 mmHg, the vessel was flushed gently to remove blood cells. The other side of the vessel was then mounted on the second cannula and also secured with a suture. The second cannula was then connected to a column filled with Krebs buffer. Pressure inside the vessel was increased in four steps to 75 mmHg. A pump (Gilson, Minipuls 3) was used to superfuse the vessel with Krebs buffer without recirculation. All vasoactive substances were applied to the vessel by adding them to the superfusate. Temperature was raised gradually to 33°C, which is the in vivo temperature of the cremaster muscle. Experiments were performed under no-flow conditions. Vessels were equilibrated for at least 30 min. At the end of each experiment the passive (maximally dilated) diameter was assessed by adding papaverine (0.1 mM). One or two segments were obtained from one animal. If two segments were used, each segment was subjected to a different protocol.

Protocol. During equilibration vessels developed spontaneous tone, leading to a ~50% reduction in diameter with respect to the passive diameter. This spontaneous tone is stable for at least several hours and avoids interference of a contractile agonist with the intervention to be studied. In the first series, the contribution of nitric oxide in the response to acetylcholine was studied. Responses to 0.1 and 0.3 µM acetylcholine were recorded before and after incubation for 30 min with Nω-nitro-L-arginine (L-NNA, 0.1 mM). Each concentration of acetylcholine was applied for at least 5 min, followed by a washout period of 20 min.

In the second series (no L-NNA present), the contribution of other factors than nitric oxide was studied. The first two groups were used to inhibit production of EDHF, whereas in the third group we focused on the mechanism of dilution. In the first group, capsaicin (1 µM) was added to deplete nerve endings of CGRP. In the second group, the role of arachidonic acid metabolites was studied. The effects of indomethacin (10 µM), a cyclooxygenase inhibitor, miconazole (1 µM) and SKF-525a (1 µM), two blockers of the cytochrome P-450 pathway, were tested on acetylcholine-induced dilatation. Likewise, in the third group the effects of high [K+], (30–50 mM), glibenclamide (1 µM), a blocker of the ATP-sensitive K channel, and TEA (1 mM), a blocker of the Ca2+-activated K+ channel, were tested. The concentration of potassium was varied to obtain a comparable increase in tone in each segment.

The response to acetylcholine was recorded before and after incubation for 20–30 min with one of these compounds. The concentration of acetylcholine used (0.1 µM) induced about half-maximal dilatation. In the experiments with miconazole, responses to nitroprusside (10 nM) were recorded to test the specificity of this inhibitor.

Statistics. The inner diameter values are reported as means ± SE (in µm). Responses to acetylcholine are changes in diameter from baseline. Because the response to acetylcholine was recorded in the presence and absence of a drug in the same arteriole, a paired Student’s t-test was used for statistical analysis. Differences were considered statistically significant as P < 0.05.

Chemicals. MOPS buffer consisted of (in mM) 145 NaCl, 2.0 KCl, 2.0 CaCl2, 1.0 MgSO4, 1.0 Na2HPO4, 5.0 dextrose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS. Krebs buffer consisted of (in mM) 110 NaCl, 5 KCl, 2.5 CaCl2, 1.2 MgSO4, 24 NaHCO3, 1.0 KH2PO4, 0.02 EDTA, and 10 dextrose. It was equilibrated with 5% CO2-21% O2 and 74% N2 at 33°C, resulting in a pH of 7.4, a P2O2 of 150 mmHg and a PCO2 of 35 mmHg (at 33°C, measured by an ABL-330, Copenhagen). A high-potassium buffer was obtained by equimolar exchange with NaCl.

RESULTS

Forty-eight arteriolar segments were studied, with a mean passive diameter of 175 ± 2 µm. After development of spontaneous tone, average diameters measured 90 ± 2 µm, reducing inner arteriolar diameter with 49 ± 1%.

Acetylcholine (0.1 and 0.3 µM) was added to the superfusate during spontaneous tone. Figure 1 shows a typical recording of a dilatory response to acetylcholine. To investigate the role of nitric oxide in the acetylcholine response, L-NNA was added to the superfusate. There was no significant effect of L-NNA on spontaneous tone (Table 1). Addition of L-NNA revealed that the response to acetylcholine consists of two components. A rapid, transient component (duration ± 2 min) resistant to L-NNA is followed by a sustained component that is virtually abolished by L-NNA (see Fig. 1). The rapid component reached its half-maximal effect in 12 ± 2 s after onset. Figure 2 shows the group data of the transient and sustained component in the response to acetylcholine and the effect of L-NNA on these components. Both components show a dose-dependent response to acetylcholine (P < 0.05). A large portion (~70%) of the first rapid component is not inhibited by L-NNA at both concentrations of acetylcholine. The second component is strongly inhibited by L-NNA and therefore likely to be mediated by nitric oxide.

![Fig. 1. Recording of a typical response to 0.3 µM acetylcholine (ACh) during spontaneous tone (top trace) and after addition of Nω-nitro-L-arginine (L-NNA, 0.1 mM) in same arteriole (bottom trace). Addition of L-NNA revealed a transient dilatory component in response to ACh that is not mediated by nitric oxide.](http://ajpheart.physiology.org/10.1152/ajpheart.00251.2005)
Table 1. Effect of inhibitors on spontaneous tone

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diameter Change, μm</th>
</tr>
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<tbody>
<tr>
<td>L-NNA</td>
<td>−5 ± 6</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>−14 ± 4†</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>−4 ± 3</td>
</tr>
<tr>
<td>Miconazole</td>
<td>22 ± 6*</td>
</tr>
<tr>
<td>SKF-525a</td>
<td>−7 ± 1‡</td>
</tr>
<tr>
<td>High potassium</td>
<td>−35 ± 4*</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>−7 ± 1‡</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>−3 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7 arterioles for Nω-nitro-L-arginine (L-NNA), n = 5 arterioles for indomethacin, and n = 6 arterioles for other compounds. Arterioles were incubated with either L-NNA (0.1 mM), capsaicin (1 µM), indomethacin (10 µM), miconazole (1 µM), SKF-525a (1 µM), high potassium 30–50 mM, glibenclamide (1 µM), or tetraethylammonium (1 mM) for 20–30 min before addition of acetylcholine. *Significant dilation (P ≤ 0.01); significant constriction †P < 0.05 or ‡P < 0.01.

The next experiments were designed to inhibit either release or effect of the transient component. In these experiments L-NNA was not present because we were interested in the interaction of the transient component with the sustained component. In the first group of the second series of experiments, the effect of capsaicin on the response to acetylcholine was tested. Capsaicin induced a transient dilation of 23 ± 10 µm, followed by an increase in tone (Table 1). A typical recording from this group is shown in Fig. 3. The transient dilation induced by capsaicin is not shown, but one should note the increase in tone. Clearly, no effect on the acetylcholine response was observed. Mean responses for the transient component are depicted in Fig. 4, A and B, for the sustained component. In the second group of this series, we tested the effect of indomethacin and two inhibitors of the cytochrome P-450 pathway, SKF-525A and miconazole. In Table 1 the effect of these compounds on spontaneous tone is shown. Indomethacin did not affect tone and miconazole induced a small dilation, whereas SKF-525a induced constriction. The effect of these compounds on the transient and sustained component of the response to acetylcholine is summarized in Fig. 4, A and B, respectively. As shown, indomethacin did not affect either component. On the other hand, both miconazole and SKF-525a dramatically inhibited the transient component. But, because the sustained component was also impaired, some concerns about the specificity of these compounds rose. However, we found no significant effect of miconazole on responses to the endothelium-independent nitric oxide donor nitroprusside (10 nM). Responses were 47 ± 7 vs. 35 ± 4 µm (n = 6) in the absence and presence of miconazole, respectively. Because SKF-525a appeared to be the most potent inhibitor of the transient component, we determined the time course of the sustained component in this group. On average, half-maximal effect of the nitric oxide-mediated dilation was reached in 3.6 ± 0.6 min.

In the last group, the effects of high potassium, glibenclamide, and TEA were tested. The effect of high potassium and the inhibitors on spontaneous tone are listed in Table 1. High potassium and glibenclamide caused further constriction, whereas TEA did not significantly affect tone. A representative recording of the effect of TEA on the response to acetylcholine is shown in Fig. 5. TEA strongly inhibited both the rapid, transient component and the sustained component. The effects of high potassium, glibenclamide, and TEA are summarized in Fig. 4, A and B. As indicated, high potassium abolished both components, whereas TEA strongly inhibited the transient component and the sustained component. In contrast, glibenclamide did not impair either component of the acetylcholine response.

![Fig. 2. Group data of first series. Transient (trans) and sustained (sust) responses to 0.1 µM (n = 7) and 0.3 µM (n = 6) ACh during spontaneous tone and after addition of L-NNA (0.1 mM) to superfusate. At both doses, transient responses are significantly impaired, whereas steady-state responses are abolished. Data are means ± SE. *P < 0.05, †P < 0.01 for L-NNA vs. spontaneous tone.](image1)

![Fig. 3. Representative recording of response to 0.1 µM ACh during spontaneous tone (top trace) and after addition of 1 µM capsaicin (bottom trace). Capsaicin induced a transient dilation (not shown) followed by an increase in tone. No effect on transient or steady-state component of the response to ACh was observed.](image2)
DISCUSSION

Two components in acetylcholine-induced dilation. The response to acetylcholine in the isolated cremaster arteriole appears as a rapid transient dilation followed by a sustained level of dilation. In the presence of L-NNA the sustained component is abolished, but a considerable part of the transient dilation in response to acetylcholine is retained. This observation confirms the findings of Koller et al. (18) who showed only a partial block of the maximal response to acetylcholine in the same preparation.

The possibility of an endothelium-derived hyperpolarizing factor mediating the transient component is supported by the results with high-potassium buffer. With 30–50 mM \( [\text{K}^+]_o \), we observed complete inhibition of the transient component. A transient EDHF-induced dilation agrees with studies that report on smooth muscle membrane potential of isolated arteries (8, 11, 19, 28). These studies showed that acetylcholine induces a transient hyperpolarization, while dilation is sustained. Thus, although membrane potential was not measured directly, our results are consistent with the hypothesis that an EDHF mediates the transient component of the response to acetylcholine. An explanation for the transient nature of EDHF has been provided by Chen and Suzuki (7). These authors presented evidence for a rapid desensitization for acetylcholine and bradykinin of the endothelial receptor that is coupled to the release of EDHF.

Candidates for EDHF. We investigated the possibility that the L-NNA resistant component is mediated by CGRP. This peptide may function as a neurotransmitter in the peripheral vasculature and hyperpolarize vascular smooth muscle. Capsaicin induces a depletion of cellular stores and is therefore a useful tool to study the role of CGRP in the acetylcholine response (3). In the in vivo cremaster muscle preparation, capsaicin induces a profound arteriolar dilation that is attributed to CGRP (16). In our study, capsaicin induced a transient dilation followed by a significant increase in tone. However, capsaicin did not affect the response to acetylcholine, indicating that CGRP is not the mediator of the L-NNA-resistant component.

Other candidates for the L-NNA resistant component are metabolites of arachidonic acid. Prostacyclin acts as a hyperpolarizing agent by opening of ATP-sensitive potassium channels (26). We have shown that inhibition of prostaglandin synthesis by indomethacin did not affect the response to acetylcholine. This result Fig. 4. A: group data of second series showing effect of inhibitors on transient response to ACh. Open bars represent control responses during spontaneous tone; filled bars represent responses in presence of indicated inhibitor. Capsaicin (1 µM) and indomethacin (10 µM) had no effect. Both cytochrome P-450 inhibitors miconazole (1 µM) and SKF-525a (1 µM) inhibited transient response. High potassium (30–50 mM) and tetraethylammonium (TEA; 1 mM), but not glibenclamide (1 µM) inhibited dilation. Data are means ± SE, with n = 5 for indomethacin and n = 6 for all other compounds. *P < 0.01 for spontaneous tone vs. inhibitor. B: group data of second series showing effect of inhibitors on steady-state response to ACh. Open bars represent control responses during spontaneous tone; hatched bars represent responses in presence of indicated inhibitor. Each compound that inhibited the peak response (A) also impaired steady-state response to ACh. Data are means ± SE, with n = 5 for indomethacin and n = 6 for all other compounds. *P < 0.01 for spontaneous tone vs. inhibitor.

Fig. 5. Typical recording of response to 0.1 µM ACh during spontaneous tone (top trace) and after addition of TEA (1 mM). TEA (bottom trace) did not significantly affect spontaneous tone but abolished transient response and impaired steady-state response to ACh.
confirms the findings of other studies on isolated arteries from various tissues, including the cremaster muscle (8, 9, 11, 12, 17). Therefore, we conclude that the transient acetylcholine-induced dilation observed after addition of L-NNA is not mediated by a prostaglandin.

To further investigate the role of arachidonic acid metabolites, we tested the effects of two cytochrome P-450 inhibitors: miconazole and SKF-525a. Both compounds induced profound inhibition of the transient component in the acetylcholine response. These results confirm the findings in other preparations that the nitric oxide-independent component in agonist-induced dilation is a cytochrome P-450 metabolite (5, 6, 14). Because miconazole selectively inhibits production of cytochrome P-450-derived EETs at the concentration used (30), these substances are the most likely candidates for EDHF. EETs induce relaxation and hyperpolarization of precontracted coronary vessels, most likely by opening of the Ca²⁺-activated K⁺ channel (5). In our study, we tested the effect of two potassium channel inhibitors. TEA, a Ca²⁺-activated K⁺ channel blocker inhibited the transient dilation, whereas glibenclamide, a blocker of the ATP-sensitive K⁺ channel was ineffective. Thus, like the EDHF in conduit arteries, the transient dilation in this study appears to be mediated by opening of Ca²⁺-activated K⁺ channels (5, 14).

Effect of inhibitors on tone. Capsaicin induced a transient dilation followed by an increase in tone. This may be explained by the release and depletion of stored CGRP, which under basal conditions could be released in small quantities. Miconazole and SKF-525a differentially affected basal tone. Miconazole induced a dilation, whereas SKF-525a induced a small constriction. Recent data suggest a role for cytochrome P-450 metabolites in tone (13) that may be differentially affected by the inhibitors used in our study. Alternatively, miconazole has been shown to inhibit L-type Ca²⁺ channels in other cell types, which may induce dilation (29). TEA has been reported to constrict isolated arteries (23). However, constriction did not reach significance in our study. Glibenclamide induced a small constriction in agreement with a report on a comparable preparation (27).

Specificity of inhibitors. Interestingly, miconazole, SKF-525a, high [K+]o, and TEA all inhibited also the nitric oxide component of the response to acetylcholine. Several potential sites of interference of these inhibitors with nitric oxide can be hypothesized. In the case of high potassium and TEA, this may be a contribution of potassium channels in nitric oxide-induced dilation (4). On the other hand, several studies argue against a role of nitric oxide on membrane potential (7, 12, 19, 28). Miconazole and SKF-525a may inhibit nitric oxide synthase because this enzyme resembles cytochrome P-450 enzymes. However, miconazole did not affect nitric oxide synthesis in brain tissue (1). Alternatively, inhibitors of the cytochrome P-450 pathway may inhibit the response to nitric oxide (20). However, we found no significant effect of miconazole on responses to the endothelium-independent nitric oxide donor, nitroprusside. A similar observation has been reported for SKF-525a (24). Thus, although a nonspecific inhibition of nitric oxide-mediated dilation cannot be ruled out, one could speculate that EDHF interferes with the release of nitric oxide (10). An EDHF may open endothelial Ca²⁺-activated K⁺ channels and cause endothelial hyperpolarization. This would increase the electrochemical gradient for Ca²⁺ and stimulate Ca²⁺-dependent nitric oxide synthesis (21).

Some reports suggest that EDHF and nitric oxide may have complementary roles. The release of EDHF was found to be inhibited by nitric oxide donors in rabbit carotid and porcine coronary arteries (2). In hypercholesterolemia, a pathological condition associated with impaired nitric oxide synthesis, the release of EETs is enhanced (25). From our experiments, we conclude that the release of these factors is largely separated in time in the acetylcholine response. This may explain why we did not observe an increase in the amplitude of the transient component in the presence of L-NNA. Our data suggest that EDHF provides a mechanism to induce a rapid dilation, which is followed by a relatively slow nitric oxide-mediated response.

In conclusion, we showed a biphasic response to acetylcholine in the cannulated cremaster artery. The first, transient component is largely mediated by a cytochrome P-450-derived metabolite, whereas the second sustained component is mediated by nitric oxide. The transient dilation is probably mediated by opening of the Ca²⁺-activated K⁺ channel.

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