Role of EDHF in conduction of vasodilation along hamster cheek pouch arterioles in vivo

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Welsh, Donald G., and Steven S. Segal. Role of EDHF in conduction of vasodilation along hamster cheek pouch arterioles in vivo. Am J Physiol Heart Circ Physiol 278: H1832–H1839, 2000.—We tested whether local and conducted responses to ACh depend on factors released from endothelial cells (EC) in cheek pouch arterioles of anesthetized hamsters. ACh was delivered from a micropipette (1 s, 500 nA), while arteriolar diameter (rest, ~40 µm) was monitored at the site of application (local) and at 520 and 1,040 µm upstream (conducted). Under control conditions, ACh elicited local (22–65 µm) and conducted (14–44 µm) vasodilation. Indomethacin (10 µM) had no effect, whereas Nω-nitro-L-arginine (100 µM) reduced local and conducted vasodilation by 5–8% (P < 0.05). Miconazole (10 µM) or 17-octadecynoic acid (17-ODYA; 10 µM) diminished local vasodilation by 15–20% and conducted responses by 50–70% (P < 0.05), suggesting a role for cyclo-oxygenase-P-450 (CYP) metabolites and cytochrome P-450 pathway (CYP) metabolites (3, 4, 7, 11, 16). Whereas each of these substances is derived from a distinct signaling pathway, all can evoke SMC hyperpolarization by augmenting K+ efflux (1, 13, 24, 27). Nevertheless, and despite extensive documentation in arterial preparations, the role of EDHF in local and conducted vasodilation in the microcirculation is undefined.

In this study, we tested the hypothesis that local and conducted vasodilation to ACh in vivo was dependent on the production of EDHF. Vasomotor responses to ACh were first evaluated in cheek pouch arterioles in the presence or absence of antagonists that inhibit the synthesis of prostaglandins (PG), NO, or CYP metabolites. Our initial findings revealed that only antagonists of the latter pathway substantively reduced local and conducted vasodilation. We then investigated the effects of two putative antagonists of CYP enzymes on the electrophysiological responses to ACh. Findings from these experiments suggest that EDHF derived from CYP activation is integral to both local and conducted vasodilation evoked by ACh in arterioles of the hamster cheek pouch.

METHODS

Our strategy was to investigate the role of each major pathway that has been implicated in the production of EDHF: cyclooxygenase, NO synthase (NOS), and CYP enzymes. Putative inhibitors of each pathway were first evaluated for their effect on vasomotor responses evoked by ACh. The electrophysiological consequences of the most effective pharmacological interventions were then investigated.

METHODS

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ure was measured using a thermocouple wire and maintained at 37–38°C with conductive heating. Using a stereo microscope (OR, Zeiss; Thornwood, NY), we exteriorized the cheek pouch onto a Plexiglas board and supercilious ical tissue was carefully removed. The preparation was superfused continuously with a bicarbonate-

buffered physiological saline solution (PSS; 37°C; pH, 7.4) of the following composition (in mM): 137.0 NaCl, 4.7 KCl, 1.2 MgSO₄, 2.0 CaCl₂, and 18.0 NaHCO₃; salts were obtained from Sigma (St. Louis, MO) or J. T. Baker (Phillipsburg, NJ) and dissolved in deionized water (dH₂O). Under control conditions, the PSS was equilibrated in a 50-ml reservoir with 5% CO₂-5% O₂, balance N₂. The preparation was placed on the stage of an intravital microscope (modified 20T; Zeiss) and transilluminated with condenser numerical aperture (NA) = 0.32 while observing through a Leitz UM 32 objective (NA = 0.20). The image was coupled to a video camera (NC-70X, Dage-MTI; Michigan City, IN) with a total magnification of ×470 at the monitor face (PVM 122, Sony; Tokyo, Japan). Dage-MTI; Michigan City, IN) with a total magnification of ×470 at the monitor face (PVM 122, Sony; Tokyo, Japan). 3A C h( 1Mi nd H₂O) was delivered from the arteriole under study; i.e., less than half the distance from the arteriole to augment dye microinjection. After recording was completed, the cell type was identified through the microelectrode to augment dye microinjection. Membrane potential, intracellular dye injection, and diameter recordings. Recording microelectrodes were pulled (P-87, Sutter Instruments; Novato, CA) from borosilicate glass tubes (GC120F-10, Warner Instruments; Hamden, CT). Tips were filled with Lucifer yellow dye (lithium salt, Sigma; 4% wt/vol solution in dH₂O; resistance ~500 MΩ); the remainder of the electrode was backfilled with 150 mM LiCl (33, 34). The reference electrode (Ag/AgCl pellet) was positioned in the effluent solution, and membrane potential (Eᵥ) was measured with an electrometer [model 773, World Precision Instruments (WPI); Sarasota, FL]. The criteria for a successful cell penetration were 1) sharp negative deflection of potential on entry; 2) stable recording of Eᵥ for at least 1 min after entry; and 3) sharp positive deflection upon exit. Cell labeling often occurred with dye diffusing from the microelectrode during the period (typically 5–20 min) of intracellular recording; in some experiments, a pulse of 100 nA was passed for 1 min through the microelectrode to augment dye microinjection. After recording was completed, the cell type was identified using epifluorescence (75 W xenon lamp; Zeiss filter set 48 77 05) while viewing through an ×40 immersion objective (NA = 0.75; Zeiss) (33, 34). Internal diameter of arterioles was measured from the monitor face using a video caliper (modified model 321; Colorado Video Instruments, Boulder, CO); spatial resolution was <2 µm. Simultaneous outputs from the electrometer and the caliper were recorded at 40 Hz using a MacLab data acquisition system (CB Sciences; Dover, NH).

Microiontophoresis. ACh (1 M in dH₂O) was delivered from a glass micropipette (∼1-µm tip; fabricated from the same glass and puller as above) using microiontophoresis. The micropipette was positioned with its tip adjacent to the abluminal surface of an arteriole using a remotely controlled hydraulic micromanipulator (MO-102, Narishige). Iontophoretic current was controlled with a programmer (model 260; WPI) gated by the MacLab system and delivered via a Ag/AgCl wire secured within the micropipette. Retain current (≥200 nA) was established as that just necessary to prevent vasodilation when the micropipette was in position.

On the basis of preliminary experiments evaluating local and conducted responses to incremental ACh pulses (200–1,000 nA; 200–2,000 ms), the stimulus selected for criterion measurements was established as 500 nA, 1 s. This stimulus consistently evoked a maximal (transient; see RESULTS) dilation at the site of stimulation that was not different from the value measured during exposure to SNP. With a 250-ms pulse (at 500 nA), both local and conducted responses were 40–50% of those obtained with the 1-s pulse. These latter responses were affected by interventions in the manner described in RESULTS; for clarity of presentation, only those data using the criterion stimulus are reported.

Vasomotor experiments. An ACh stimulus was delivered onto a second- or third-order arteriole while vasomotor responses were monitored at the “local” site of stimulation (distance = 0 µm) and at conducted sites located 520- and 1,040-µm upstream along the arteriole as measured with a calibrated eyepiece reticule. Responses at these distances were obtained by repeating an identical stimulus for each observation (18, 28, 34); the vessel was allowed to recover to resting diameter for 2–3 min between successive stimuli. Extensive control experiments have established that there is negligible tachyphylaxis to ACh using this protocol and that diffusion of ACh from the stimulus site is negligible for distances greater than ~100 µm (18, 28, 34). As an internal control throughout these experiments, the micropipette containing ACh was positioned in the tissue at a site ≈250 µm from the arteriole under study; i.e., less than half the distance observed for the nearest site of conduction. When an ACh pulse was delivered from these sites, there was no effect on arteriolar diameter, confirming that neither diffusion nor convection of ACh could explain conducted responses.

Vasomotor responses to ACh were measured under control conditions or following superfusion (~20 min) with one of the following agents added to the PSS: indomethacin (10 µM, Research Biochemicals International; Natick, MA) to inhibit cyclooxygenase; N-nitro-l-arginine (L-NAME, 100 µM; Sigma) to inhibit NOS in the absence and presence of excess l-arginine (1 mM; Sigma); and either miconazole (10 µM; Cayman; Ann Arbor, MI) or 17-ocytadecynoic acid (17-ODYA; 10 µM, Cayman) to inhibit CYP enzyme activity (14). Stock solutions of indomethacin, miconazole, and 17-ODYA were dissolved in ethanol; L-NNA and L-arginine were dissolved in PSS. Ethanol vehicle controls (0.2–0.5% in PSS) had no effect on resting diameter and did not influence either local or conducted responses to ACh (data not shown).

Whereas L-NNA increases basal tone (29), we have shown that vasoconstriction per se does not alter conducted vasodilation (19, 28, 29). The concentrations of inhibitors used here were based on our own studies (29) and values reported by others (17) studying the microcirculation. For treatment with 17-ODYA, a preparation was superfused with the inhibitor for 40–45 min. During this period, O₂ in the superfusate was cycled between 5% and 21% (with 5% CO₂, balance N₂) every 10 min, and the arteriole was stimulated with ACh (500 nA, 1 s) every 5 min. Previous studies have shown that both O₂ and ACh augment the activity of CYP enzymes (14, 23). Because 17-ODYA will bind only to activated CYP enzymes (14), it was reasoned that this protocol would augment the efficacy of inhibition. We have observed progressive diminution in arteriolar responses to the cycling of oxygen during this protocol (J. Hungerford and S. Segal, unpublished observations). Before the effects of 17-ODYA on responses to ACh were evaluated, the preparation was reequilibrated for 15 min with control PSS to eliminate unbound inhibitor and minimize nonspecific effects (14). The stability of control responses to ACh exceeded 60 min.

Electrophysiological experiments. On the basis of the effects of respective inhibitors on vasomotor responses (see RESULTS),
additional experiments were designed to test whether CYP inhibition impaired local and conducted hyperpolarization to ACh. A modified stimulus protocol enabled local and conducted responses to be evaluated at the same site along the arteriole (34). First, the direct (local) vasomotor response to ACh was recorded. Then, with the use of the remotely controlled micromanipulator, ACh was delivered onto the arteriole at 520 µm downstream to obtain the conducted vasomotor response. Once these control data were obtained, the preparation was treated with either miconazole or 17-ODYA as above, or control conditions were maintained. A recording microelectrode, secured in a single-axis hydraulic micromanipulator (MX510, Siskiyou Design Instruments; Grants Pass, OR) and aligned with the vessel axis, was then carefully lowered onto the edge of the arteriole, and a cell was penetrated at an angle of ~60° (33, 34). Once a stable E_m and vessel diameter were attained (~1 min), ACh was released at the recording site and the local responses were obtained. The stimulus was repeated at 520 µm downstream to obtain the conducted responses (34). Vasomotor and E_m were recorded simultaneously under respective conditions. At the end of an experiment, the cell type recorded from was identified by the pattern of fluorescent dye labeling: individually labeled SMC wrapped around the arteriole, whereas dye-coupled EC aligned with the vessel axis (33, 34).

Data presentation and analysis. Summary data are presented as means ± SE. In Figs. 1 and 2, the change in diameter in response to ACh was calculated as the difference between rest and peak values. Paired t-tests were used to compare ACh responses at each location under control conditions to those during treatment with an inhibitor. In Figs. 3 and 5, representative recordings of E_m are shown with corresponding vasomotor responses to illustrate the effects of pharmacological interventions. Because the effective duration of intracellular recordings precluded paired measurements in the same cell before and after pharmacological interventions, EC and SMC responses for respective conditions within each figure are from separate experiments. In Figs. 4 and 6, the "percentage of initial diameter response" was calculated as the change in diameter during intracellular recording divided by the corresponding change in diameter before intracellular recording or intervention. Corresponding

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Fig. 1. Effects of 10 µM indomethacin (A, n = 5) or 100 µM N-nitro-L-arginine (L-NNA; B, n = 9) on arteriolar dilation to ACh (500 nA, 1 s). Diameter was measured at site of stimulation (distance = 0 µm) and at 520 and 1,040 µm upstream from stimulus. Arteriolar diameters (µm) were as follows. A: control, 43 ± 4; indomethacin, 42 ± 4; indomethacin + 10 µM sodium nitroprusside (SNP), 82 ± 5. B: control, 39 ± 2; L-NNA, 30 ± 2; L-NNA + 10 µM SNP, 76 ± 3. *Significantly different from control (P < 0.05).

Fig. 2. Effects of 10 µM miconazole (A, n = 6) or 10 µM 17-octadecynoic acid (17-ODYA; B, n = 13) on arteriolar dilation to ACh (500 nA, 1 s). Diameter was measured at site of stimulation (distance = 0 µm) and at 520 and 1,040 µm. Arteriolar diameter (µm): A: control, 32 ± 5; miconazole, 34 ± 3; miconazole + 10 µM SNP, 64 ± 7. B: control, 31 ± 2; 17-ODYA, 33 ± 2; 17-ODYA + 10 µM SNP, 75 ± 6. *Significantly different from control (P < 0.05).
Table 1. Effect of 17-ODYA alone and in combination with L-NNA on vasodilation to acetylcholine

<table>
<thead>
<tr>
<th>Distance, µm</th>
<th>Change in Diameter, µm</th>
<th>Control</th>
<th>17-ODYA</th>
<th>17-ODYA + L-NNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>42 ± 4</td>
<td>33 ± 3*</td>
<td>15 ± 2†</td>
<td></td>
</tr>
<tr>
<td>520</td>
<td>25 ± 2</td>
<td>12 ± 1*</td>
<td>8 ± 1‡</td>
<td></td>
</tr>
<tr>
<td>1,040</td>
<td>22 ± 2</td>
<td>10 ± 1*</td>
<td>6 ± 1‡</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Vasodilator responses (i.e., change in diameter) of arterioles (n = 13) to ACh microiontophoresis (500 nA, 1 s) were monitored under control conditions and in the presence of 10 µM 17-octadecynoic acid (17-ODYA) alone or in combination with 100 µM N^**-nitro-L-arginine (L-NNA; see METHODS for details). Responses were evaluated at the site of stimulation (distance = 0 µm) and at 520 and 1,040 µm upstream along the arteriole. Diameter (µm): control, 31 ± 2; 17-ODYA, 33 ± 2; 17-ODYA + L-NNA, 25 ± 2. Control and 17-ODYA data are shown in Fig. 2B and included here for reference. †Significantly different from control and from 17-ODYA alone, respectively (P < 0.05).

RESULTS

Effects of indomethacin and L-NNA. ACh microiontophoresis evoked vasodilation locally (amplitude, 37 ± 3 µm) that was conducted 520 (23 ± 2 µm) and 1,040 µm (19 ± 1 µm) upstream (Fig. 1A). Typically, 2–3 s elapsed between the stimulus and the onset of vasodilation, with no discernable difference in the onset of dilation with distance along the arteriole. These responses were unaffected by the addition of indomethacin to the superfusate. In contrast, L-NNA reduced resting diameter by 8–10 µm (P < 0.05) and attenuated both local and conducted responses to ACh by 2–5 µm (Fig. 1B). The addition of 1 mM L-arginine reversed the increase in resting tone [diameter (µm): control, 38 ± 2; L-NNA, 30 ± 3; L-NNA + L-arginine, 35 ± 3] and restored vasodilation to ACh at the local site [response (µm): control, 35 ± 2; L-NNA, 30 ± 3; L-NNA + L-arginine, 35 ± 2 (n = 7)].

Effects of miconazole and 17-ODYA. Treatment with miconazole or 17-ODYA had no significant effect on either resting diameter of arterioles or their responses to SNP (Fig. 2). Both inhibitors significantly reduced vasodilations to ACh (Fig. 2, legend), with much greater effects on conducted responses (50–70%) than on local responses (15–20%). Addition of L-NNA following 17-ODYA inhibited local responses by an additional 43% and conducted responses by an additional 15–20% (Table 1).

Electrophysiological responses. The pronounced effects of either miconazole or 17-ODYA on conducted vasodilation suggested that CYP metabolites were produced by EC in response to ACh (4, 14), and that these products caused hyperpolarization of surrounding SMC (4, 34). This hypothesis was investigated by recording electrophysiological responses of SMC and EC to ACh in the absence and presence of either inhibitor. Under control conditions, ACh elicited hyperpolarization of SMC (Figs. 3 and 4) and EC (Figs. 5 and 6) that conducted along the arteriolar wall; the onset of electrical responses preceded the onset of local and conducted vasodilation by 1–2 s. Miconazole inhibited the hyperpolarization of both SMC (7 of 8 cells) and EC (6 of 7 cells). In contrast 17-ODYA inhibited hyperpolarization of SMC (7 of 10 cells) with no effect on EC hyperpolarization (5 of 5 cells).

DISCUSSION

In arterioles of the hamster cheek pouch, ACh initiates a vasodilation that conducts along the vessel wall and has been attributed to the spread of hyperpolarization from cell to cell through gap junctions (21, 28, 34). The apparent lack of electrical coupling between EC and SMC layers in vivo (33, 34) led us to determine here whether endothelium-derived factors contribute to local and conducted vasomotor and electrical responses. First, arteriolar diameter responses to ACh were studied in the absence and presence of agents that inhibit...
the synthesis of prostaglandins, NO, and CYP metabolites, respectively. These experiments revealed that inhibition of cyclooxygenase had no measurable effect on local or conducted vasodilation and that NOS blockade had a modest effect, reducing responses by <10%. In contrast, inhibition of the CYP enzymes attenuated local vasodilation by 15–20% and conducted vasodilation by 50–70%. Experiments in which E_m was recorded from identified cells revealed that miconazole blocked hyperpolarization in EC as well as SMC; these effects likely reflect nonselective K^+ channel inhibition by this CYP antagonist (10, 15). In contrast, 17-ODYA selectively inhibited ACh-evoked hyperpolarization of SMC without affecting that of EC, which is consistent with the actions of an EDHF (13, 14). These findings indicate that products of the CYP pathway in EC contribute substantively to local and conducted vasodilation in cheek pouch arterioles by hyperpolarizing surrounding SMC.

Effects of cyclooxygenase and NOS inhibition on vasomotor responses. In muscular arteries, ACh-induced relaxation of SMC reflects, at least in part, the activation of cyclooxygenase (26) and NOS (12, 31). To examine whether the synthesis of prostaglandins or of NO is integral to local or conducted vasodilation in arterioles, responses to ACh microiontophoresis were monitored under control conditions and in the presence of indomethacin or L-NNA, respectively. As shown in Fig. 1, neither resting tone nor the effector pathway(s) activated by ACh were dependent on vasodilatory prostanoids (e.g., prostacyclin). In comparison, the reduction in resting diameter with L-NNA (with reversal of this effect by excess L-arginine) indicated a constitutive role for NO in modulating basal tone in vivo. Both local and conducted vasodilation were attenuated by treatment with L-NNA. This modest (<10%) effect is consistent with the findings that NOS inhibition had little effect on the local and conducted responses to such brief ACh stimuli as used in the present study (9, 29). We
Therefore conclude that, whereas NO may indeed function as an EDHF (25), its production is not integral to the conduction of vasodilation evoked by ACh in arterioles of the hamster cheek pouch.

Effects of cytochrome P-450 inhibitors on vasomotor and electrophysiological responses. The CYP enzymes are a complex family of proteins that produce biologically active metabolites from arachidonic acid (4, 14). In the resistance vasculature, metabolites such as epoxyeicosatrienoic and hydroxyeicosatetraenoic acids can regulate vessel diameter by influencing the activity of the Ca^{2+}-activated K⁺ channel in SMC (3, 20). We reasoned that if arteriolar dilation to ACh is indeed dependent on such factors, then CYP antagonists should attenuate the local and conducted hyperpolarization in SMC. As shown in Fig. 2, either miconazole or 17-ODYA significantly reduced arteriolar dilations in response to ACh, particularly at the conducted sites. The conduction of vasodilation ultimately reflects the spread of hyperpolarization into and along SMC (34). Thus the >50% inhibition of electrical responses observed here suggests that CYP metabolites are hyperpolarizing factors that contribute in part (15–20%) to local vasodilation and are integral to the corresponding conducted response. A role for CYP metabolites in ACh-induced vasodilation has been shown for several mammalian species (7, 16), including the hamster microcirculation (6a). The present data are the first to illustrate their role in the conduction of vasodilation along arterioles during blood flow control. Nevertheless, it is clear that CYP antagonists are not efficacious in all vascular beds (32, 35), which may be attributed to regional differences in the expression of these enzymes (4).

Implicit in the preceding argument is the assumption that such inhibitors as miconazole and 17-ODYA act specifically to inhibit the CYP pathway. Such assumptions, however, should be viewed with caution, because these agents may block hyperpolarization by inhibiting voltage-dependent, ATP-sensitive, and Ca^{2+}-activated K⁺ channels (10, 15). Indeed, the possibility that miconazole is directly blocking K⁺ channel conductance is supported by its inhibition of EC hyperpolarization to ACh (Figs. 5 and 6). This signaling pathway has been shown to reflect the stimulation of Ca^{2+}-activated K⁺ channels through G proteins (6), which occurs independent of endothelium-derived factors. Thus blockade of EC hyperpolarization to ACh with miconazole reveals nonselective actions of this antimicrobial agent in cheek pouch arterioles. In contrast, the maintenance of EC hyperpolarization during inhibition of both SMC hyperpolarization and conducted vasodilation with 17-ODYA indicates greater specificity of this suicide substrate and argues further against prevalent electrical coupling between EC and SMC in cheek pouch arterioles in vivo (33, 34).

The inhibition of a single endothelial-derived product did not completely block ACh-induced dilation in cheek pouch arterioles (Figs. 1 and 2). This was particularly evident at the local site, where NOS and CYP antagonists alone attenuated the response to ACh by <10% and 15–20%, respectively. Furthermore, local hyperpolarizations were blocked to a greater extent than the associated vasodilations (Figs. 3 and 4). These findings indicate that the direct effect of ACh on arterioles involves the release of several endothelial-derived factors that relax SMC through pharmacomechanical as well as electromechanical coupling (30). This interpretation is consistent with reports on muscular arteries, where EC release prostanoids, NO, and at least one additional factor (possibly a CYP metabolite) in response to muscarinic receptor activation (3, 5, 7, 11). In our experiments where L-NNA was applied in combination with 17-ODYA (Table 1), responses to ACh were more effectively blocked than with either agent alone, with a synergistic effect apparent at the local site.

We did not measure electrophysiological responses in the presence of L-NNA. Nevertheless, indirect evidence suggests that NO relaxes SMC in cheek pouch arterioles primarily through pharmacomechanical coupling.
For example, when SNP (an NO donor) is released as a pulse onto a cheek pouch arteriole, a local dilation occurs that is similar in magnitude to that observed with ACh (18). However, the lack of a conducted response to SNP argues against the initiation of hyperpolarization. Indeed, if NO was an effective hyperpolarizing factor in cheek pouch arterioles, then l-NNA would be expected to block conducted dilation in the manner shown for 17-ODYA (Fig. 2B) and this did not occur. Even when combined with 17-ODYA, the additional effect of l-NNA on conducted responses was modest (Table 1). From the present findings, experiments using a complement of inhibitors and range of stimulus parameters are required to resolve respective components of electrical and mechanical responses to ACh (and other stimuli) at local and conducted sites.

In conclusion, ACh has proven to be a powerful tool in elucidating the nature of paracrine factors that are released from EC and the mechanisms by which these factors effect SMC relaxation. ACh has been similarly effective as a stimulus for defining the properties of conducted vasodilation in arterioles. The present study is the first to investigate a role for EDHF in arteriolar responses to ACh using intracellular recording from defined EC and SMC. The absence of an effect of indomethacin and the modest effect of l-NNA indicate that neither prostaglandins nor NO are essential to the induction or the conduction of vasodilation in hamster cheek pouch arterioles. In contrast, the pronounced attenuation of conducted vasodilation by 17-ODYA, together with its inhibition of SMC (but not EC) hyperpolarization to ACh, indicate that the activation of muscarinic receptors on arteriolar EC evokes the rapid (<1 s) release of CYP metabolites that hyperpolarize and relax the surrounding SMC. These findings from in vivo experiments provide unique evidence for the role of EDHF during blood flow control to peripheral tissue.

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