Nitric Oxide–Independent Relaxations to Acetylcholine and A23187 Involve Different Routes of Heterocellular Communication

Role of Gap Junctions and Phospholipase A2

Iain R. Hutcheson, Andrew T. Chaytor, W. Howard Evans, Tudor M. Griffith

Abstract—NO- and prostanoid-independent relaxations are generally assumed to be mediated by an endothelium-derived hyperpolarizing factor (EDHF) that has been postulated to be an arachidonic acid metabolite. Recent evidence also suggests that direct heterocellular gap junctional communication (GJC) between endothelium and smooth muscle contributes to NO-independent relaxations. In the present study we have investigated the contribution of phospholipase A2 (PLA2)-linked metabolites and GJC to EDHF-type relaxations in rabbit mesenteric artery. In isolated rings preconstricted with 10 μmol/L phenylephrine in the presence of N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME) and indomethacin, acetylcholine (ACh) and the Ca\textsuperscript{2+} ionophore A23187 evoked relaxations that were markedly attenuated by the Ca\textsuperscript{2+}-dependent PLA2 inhibitors 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid (3 μmol/L) and arachidonyl trifluoromethyl ketone (3 μmol/L), but were potentiated by the sulfhydryl agent thimerosal (300 nmol/L). In intact rings, relaxations to ACh were attenuated synergistically by L-NAME and Gap 27 peptide, an inhibitor of GJC, whereas ACh-evoked relaxations of "sandwich" preparations were unaffected by the peptide but were abolished by L-NAME. In both ring and sandwich preparations A23187-induced relaxations were attenuated by inhibition of PLA2 but were insensitive to L-NAME and Gap 27 peptide. We conclude that EDHF-type relaxations of rabbit mesenteric artery to ACh and A23187 depend on a common pathway that involves activation of PLA2. In the case of ACh, relaxation requires transfer of a factor or factors from the endothelium to smooth muscle via gap junctions, whereas A23187 permits release directly into the extracellular space. (Circ Res. 1999;84:53-63.)

Key Words: EDHF ■ phospholipase A\textsubscript{2} ■ gap junction ■ acetylcholine ■ A23187

The endothelium plays a central role in the control of vascular tone through the release of vasoactive autacoids in response to agonist stimulation and shear stress. Mediators include NO, prostanoids, and in many vessel types a distinct endothelium-derived hyperpolarizing factor (EDHF). The chemical identity of EDHF remains controversial, although it seems probable that it mediates smooth muscle hyperpolarization by activating K\textsuperscript{+} channels (see References 2 and 3 for review). Recent reports suggest that epoxyeicosatrienoic acids (EETs), which are cytochrome P-450 monoxygenase metabolites of arachidonic acid, and the endocannabinoid anandamide, which is also derived from arachidonic acid, possess the characteristics of EDHF in certain artery types. It has been suggested that the source of arachidonic acid for EDHF production is the Ca\textsuperscript{2+}-dependent cytosolic form of phospholipase A\textsubscript{2} (PLA\textsubscript{2}), since specific inhibitors of this enzyme attenuate NO-independent relaxations to acetylcholine (ACh), histamine, and bradykinin in the perfused rat heart and mesenteric bed. EDHF has been detected both in cascade bioassay and in sandwich preparations, suggesting that this factor can diffuse freely in the extracellular space. However, recent evidence suggests that NO-independent relaxations in rabbit mesenteric artery and aorta could involve the preferential direct transfer of a mediator through gap junctions, rather than via a common extracellular route. Intercellular continuity is facilitated by gap junctions that are assembled when 2 connexon hemichannels supplied by neighboring cells interact and dock. Each connexon consists of 6 connexin protein subunits arranged around a central pore, and the characteristic pentalaminar appearance of sections of gap junction plaques has previously been demonstrated in rabbit conduit arteries. Connexin 43 is the most prevalent subtype in endothelium-denuded rabbit superior mesenteric artery, and connexins 37, 40, and 43 have been identified in endothelial cells. Previous studies have shown that the inhibitory Gap 27 peptide (amino acid sequence SRPTEKTIFII), which possesses conserved sequence homology with a region of the...
second extracellular loop of these connexins, rapidly and reversibly attenuates NO-independent relaxations to the agonists ACh and adenosine triphosphate and also to cyclopiazonic acid. Direct heterocellular communication may therefore contribute to EDHF-mediated smooth muscle relaxations evoked by receptor-dependent and -independent activation of the endothelial cell. Although the exact mode of action of Gap 27 peptide remains to be elucidated at the molecular level, possibilities include (1) inhibition or reversal of connexon docking before or after penetration of the peptide into the intercellular gap and (2) induction of a conformational change that results in channel closure.

In the present study we have used isolated ring and sandwich preparations to evaluate the contributions of gap junctional communication (GJC) and PLA₂ to NO-independent relaxations evoked by ACh and the calcium ionophore A23187 in rabbit superior mesenteric artery. Arachidonyl trifluoromethyl ketone (AACOCF₃), an inhibitor of the cytosolic form of PLA₂; [E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-1] (HELSS), a specific inhibitor of the secretory form of PLA₂; 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RO-082), an inhibitor of both forms of PLA₂; 1-(6-[[17β-3-methoxyestra-1,3,5(10)-tiene-17-y]laminio]hexyl)-1H-pyrrole-2,5-dione (U-73122), a specific inhibitor of phospholipase C (PLC); and thimerosal, an organic mercury sulfhydryl reagent that is known to inhibit the acyl-coenzyme A:lysocetlin acyltransferase and to modulate mobilization of intracellular Ca²⁺ from stores, were used to characterize the pathways involved in the mediation of these relaxations. Gap 27 peptide was used to elucidate the predominant diffusion pathway of the putative mediator once formed. Previous studies have shown that this peptide does not directly affect ambient force development in the rabbit mesenteric artery.

Materials and Methods

Isolated Ring Preparations

Experiments were performed with superior mesenteric arteries from male New Zealand White rabbits (2.5 kg) that had been sacrificed by injection of sodium pentobarbitate (120 mg/kg IV). The tissues were transferred to cold Holman’s solution of the following composition (in mmol/L): NaCl 120, KCl 5, Na₂HPO₄ 1.3, NaHCO₃ 25, CaCl₂ 2.5, glucose 11, and sucrose 10. Rings (2 to 3 mm wide) were cut and suspended by thread from FT102 force transducers connected to a MacLab/4e (ADInstruments) in 3-mL organ baths containing gassed (95% O₂ and 5% CO₂, pH 7.4) Holman’s solution at 37°C to measure isometric force development. The rings were placed under a resting tension of 0.5 g and allowed to equilibrate for ~1 hour with frequent readjustment of tension to allow for stress relaxation.

Experimental Protocol

The rings were precontracted with 10 μmol/L phenylephrine, and cumulative concentration-response curves to ACh (1 nmol/L-10 μmol/L) and the Ca²⁺ ionophore A23187 (0.1 nmol/L-1 μmol/L) were constructed before and after a 45-minute incubation with N⁶-nitro-l-arginine methyl ester (L-NAME) (300 μmol/L) alone or a combination of L-NAME and 1H-(1,2,4)oxadiazolo-(4,3a)-quinazoline-1-1 (ODQ, 10 μmol/L), an inhibitor of guanylate cyclase. Experiments were also performed in the presence of indomethacin (10 μmol/L) to determine the involvement of prostanoïd synthesis. The role of GJC was investigated by preincubating for 20 minutes with Gap 27 peptide (300 μmol/L). To analyze the role of PLA₂ and PLC in the L-NAME–sensitive and –insensitive components of the relaxations, cumulative concentration-response curves were constructed following a 45-minute incubation with ONO-RO-082 (1 to 10 μmol/L), AACOCF₃ (3 μmol/L), HELSS (300 nmol/L), or U-73122 (500 nmol/L), respectively. In other studies, thimerosal (300 nmol/L) was added 20 minutes before construction of concentration-response curves to ACh and A23187 in the absence and presence of L-NAME. These experiments were then repeated in the presence of Gap 27 peptide (300 μmol/L).

To assess the vasoactivity of EETs in this preparation, cumulative concentration-response curves to 5,6-, 11,12-, and 14,15-EET (100 nmol/L to 3 μmol/L) were constructed in the absence and presence of L-NAME. In studies in which relaxation was observed, the experiments were repeated in the presence of Gap 27 peptide. Responses to ACh and A23187 were also investigated before and after a 30-minute incubation with 5 μmol/L of each of the 3 EET isomers.

Sandwich Experiments

In a separate series of experiments, rings of superior mesenteric artery were cut open and small strips of endothelium intact vessel were sutured onto strips of endothelium-denuded artery, with their intimal surfaces in close apposition, to provide composite preparations. These sandwich preparations were allowed to equilibrate to a tension of 0.6 g for ~1 hour before precontraction with phenylephrine (10 μmol/L), with tension monitored only in the endothelium-denuded vessel. The transferable nature of endothelium-dependent relaxations to ACh and A23187 were assessed in the absence and presence of L-NAME (300 μmol/L), ONO-RO-082 (3 μmol/L), and Gap 27 peptide (300 μmol/L).

Materials

ACh, phenylephrine, indomethacin, thimerosal, A23187, and L-NAME were obtained from Sigma. U-73122 was obtained from Calbiochem-Novabiochem; ONO-RO-082 was obtained from Alexis Corp; and 5,6-EET, 11,12-EET, 14,15-EET, HELSS, AACOCF₃, and ODQ were obtained from Affiniti Research Products Ltd. Gap 27 peptide (SRPTEKTIFII) was synthesized by Severn Biotech Ltd. All drugs were dissolved in Holman’s buffer with the exception of thimerosal (300 nmol/L) was added 20 minutes before construction of concentration-response curves to ACh and A23187 in the absence and presence of L-NAME (300 μmol/L), ONO-RO-082 (3 μmol/L), and Gap 27 peptide (300 μmol/L).

Statistics

All data are given as mean±SEM, and n denotes the number of animals studied for each data point. Concentration-response curves were assessed by 1-way ANOVA followed by the Bonferroni multiple comparisons test. EC₅₀ and maximal responses were compared by the Student’s t test for paired and unpaired data as appropriate. P<0.05 was considered as significant.

Results

Effects of PLA₂ and PLC Inhibition on ACh- and A23187-Induced Relaxations in Intact Rings

ACh and A23187 both evoked endothelium-dependent relaxations of mesenteric rings that were maximal at ~3 μmol/L and ~300 nmol/L, respectively. Addition of L-NAME (300 μmol/L) to the organ bath increased tone by 18.8±1.5% (n=20) and attenuated ACh-induced relaxations, causing an increase in the EC₅₀ value from 0.16±0.02 to 0.76±0.11 μmol/L (P<0.001, n=25) and a reduction in the maximal response (control, 70.9±2.8%; L-NAME, 26.4±2.7%; P<0.001, n=25). The EC₅₀ value for relaxation to A23187 was similarly increased from 50.0±1 to 134.5±30 nmol/L following incubation with L-NAME (n=21), and there was a smaller but significant reduction in maximal
A23187-induced relaxations (Figure 1). Similarly, indomethacin had no further inhibitory effect on either ACh- or RS-082 (3 μmol/L) relaxations to ACh or A23187 both in the absence and presence of L-NAME (Figure 1). Indomethacin was included in the buffer for all remaining studies. Additionally, ODQ (10 μmol/L) did not modulate the L-NAME–insensitive relaxations to A23187 (B) either in terms of the EC50 value (control, 0.18±0.03 μmol/L; HELSS, 0.27±0.07 μmol/L; n=4) or maximal response (control, 23.1±5.1%; HELSS, 24.4±6%; n=4). Similarly, HELSS was without effect on the L-NAME–insensitive responses to A23187, with EC50 values (control, 108±21 nmol/L; HELSS, 93.5±32 nmol/L; n=4) and maximal responses (control, 66.5±3.4%; HELSS, 60.5±9.5%; n=4) being unchanged.

Preincubation with U-73122 (500 nmol/L), a specific PLC inhibitor, in the presence of L-NAME attenuated relaxations to ACh, with EC50 values being increased from 0.36±0.12 to 1.06±0.3 μmol/L and maximal relaxations reduced from 29.6±7.6% to 10.2±3.1% (P<0.05, n=5, Figure 2C). U-73122 had no significant effect on the L-NAME–insensitive response to A23187 (Figure 2D).

**Effects of Gap 27 Peptide on ACh- and A23187-Induced Relaxations in Intact Rings**

Preincubation of rings with Gap 27 peptide (300 μmol/L) had no effect on phenylephrine-induced tone (not shown) but significantly attenuated relaxations to ACh, reducing the maximal response from 81.3±4% to 58.8±5.5% (P<0.01, n=8) and causing a rightward shift in the concentration–response curve and an increase in the EC50 value from 200±20 to 480±80 nmol/L (P<0.01, n=8, Figure 3A). By contrast, A23187-induced relaxations were completely unaffected by pretreatment with Gap 27 peptide (n=4, Figure 3B), with no change in either EC50 values (52±5 to 50±4 nmol/L) or maximal responses (75.9±6% to 67.5±2%). In the presence of L-NAME (300 μmol/L), Gap 27 peptide significantly attenuated the residual relaxation to ACh, causing a further increase in the EC50 value from 281±93 to 428±52 nmol/L (n=4) and a significant reduction in the maximal response from 54±3.2% to 33.3±4.9% (P<0.05, n=4, Figure 3A). Gap 27 peptide was without effect on the L-NAME–insensitive component of the relaxations to A23187 either in terms of the EC50 values (54±6 to 58±8 nmol/L, n=4) or maximal response (61.5±1.3% to 61.8±4.1%) (n=4, Figure 3B). Figure 3C illustrates that for relaxations greater than ~20%, the time taken from onset to relaxation from 73.4±3.4% to 61.1±2% (n=21, P<0.01). These values were obtained by pooling data from all experiments. Addition of ODQ (10 μmol/L) in the presence of L-NAME had no further inhibitory effect on either ACh- or A23187-induced relaxations (Figure 1). Similarly, indomethacin was without effect on the EC50 values and maximal relaxations to ACh or A23187 both in the absence and presence of L-NAME (Figure 1). Indomethacin was included in the buffer for all remaining studies.

In the absence of L-NAME, inhibition of PLA2 with ONO-RS-082 (3 μmol/L) had no effect on ACh-induced relaxations (Figure 2A) in terms of either the EC50 value (control, 0.18±0.05 μmol/L; ONO-RS-082, 0.18±0.09 μmol/L; n=5) or maximal relaxations (control, 73.5±2.4%; ONO-RS-082, 72.7±4.2%; n=5) but increased the EC50 values for A23187 from 50±0.1 to 340±80 nmol/L (P<0.01, n=4, Figure 2B), although there was no significant effect on maximal relaxations (control, 70.2±3%; ONO-RS-082, 64.5±3.3%). There was also no effect of AACOCF3 (3 μmol/L) on ACh-induced relaxations in terms of either the EC50 value (control, 0.16±0.01 μmol/L; AACOCF3, 0.13±0.03 μmol/L; n=3 for both) or maximal relaxations (control, 70.8±4.7%; AACOCF3, 72.5±2.1%; n=3 for both), but this agent significantly increased the EC50 value for A23187 from 51±0.3 to 122±29 nmol/L (P<0.05, n=3) and caused a small but not significant reduction in the maximal response from 67.3±2.9% to 53.3±7.9% (n=3). In the presence of L-NAME, ONO-RS-082 almost abolished ACh-induced relaxations, with maximal responses being reduced from 37.7±4.6% to 8.8±3% (P<0.01, n=8, Figure 2A). AACOCF3 also virtually abolished relaxations to ACh, with maximal responses being reduced from 26.4±3.2% to 6.6±1.4% (P<0.01, n=4, Figure 2A). Furthermore, both ONO-RS-082 and AACOCF3 significantly attenuated the maximal L-NAME–insensitive relaxations to A23187 from 54.2±7% to 23.4±7.2% (P<0.01, n=5, Figure 2B) and from 66.5±3.4% to 27.3±7.8% (P<0.05, n=6, Figure 2B), respectively. HELSS (300 nmol/L) had no effect on ACh-induced relaxations in the presence of L-NAME either in terms of the EC50 value (control, 0.18±0.03 μmol/L; HELSS, 0.27±0.07 μmol/L; n=4) or maximal response (control, 23.1±5.1%; HELSS, 24.4±6%; n=4). Similarly, HELSS was without effect on the L-NAME–insensitive responses to A23187, with EC50 values (control, 108±21 nmol/L; HELSS, 93.5±32 nmol/L; n=4) and maximal responses (control, 66.5±3.4%; HELSS, 60.5±9.5%; n=4) being unchanged.

Preincubation with U-73122 (500 nmol/L), a specific PLC inhibitor, in the presence of L-NAME attenuated relaxations to ACh, with EC50 values being increased from 0.36±0.12 to 1.06±0.3 μmol/L and maximal relaxations reduced from 29.6±7.6% to 10.2±3.1% (P<0.05, n=4, Figure 2C). U-73122 had no significant effect on the L-NAME–insensitive response to A23187 (Figure 2D).

**Figure 1.** Concentration-response curves for endothelium-intact rings of rabbit mesenteric artery showing that indomethacin (10 μmol/L) did not modulate relaxations to ACh (A) and A23187 (B) either in the absence (n=5 for both agents) or presence (n=4 for both agents) of L-NAME (300 μmol/L). Furthermore, ODQ (10 μmol/L) did not modulate the L-NAME–insensitive relaxations to either ACh (A) or A23187 (B) (n=3 for both agents).
maximum relaxation was substantially longer for A23187 than for ACh in the presence of L-NAME.

Correlations With the Initial Relaxant Response to ACh

Figure 4 illustrates that in any given preparation there was an inverse relationship between the magnitude of the initial relaxations to ACh and those subsequently obtained in the presence of L-NAME. Linear regression analysis confirmed a negative correlation between maximal responses to ACh in the presence and absence of L-NAME (P<0.01, n=19, r=0.60). There was a direct relationship between the magnitude of the initial relaxations to ACh and those subsequently obtained in the presence of Gap 27 peptide, with a positive correlation coefficient being confirmed by linear regression analysis (P<0.001, n=8, r=0.94).

Effects of Thimerosal on ACh- and A23187-Induced Relaxations in Intact Rings

Thimerosal (300 nmol/L) did not affect phenylephrine-induced tone (not shown). Incubation of rings for 20 minutes with this agent, in the absence of L-NAME, enhanced relaxations to ACh (Figure 5A), with a significant reduction in EC50 values from 0.11±0.03 µmol/L to 21±0.3 nmol/L (P<0.05, n=5) but no effect on maximal responses (control, 73.5±5.5%; thimerosal, 76.6±5.3%; n=5). Gap 27 peptide (300 µmol/L) had no effect on the thimerosal-induced enhancement in terms of the EC50 value (25±0.4 nmol/L, n=4) but significantly reduced the maximal relaxations to 51.9±2.5% (n=4). Similarly, A23187-induced relaxations were enhanced by thimerosal in the absence of L-NAME (Figure 5B), with EC50 values being reduced from 70±9 nmol/L to 33±10 nmol/L (P<0.05, n=4) and the maximal response showing a small, but not significant, rise from 75.1±8.7% to 86.2±4.3% (n=4). In the presence of L-NAME, thimerosal significantly reduced the EC50 values for both ACh (from 0.51±0.13 to 0.13±0.05 µmol/L, P<0.05, n=10) and A23187 (from 0.18±0.06 to 0.083±0.03 µmol/L, n=6, Figure 5C and 5D). Furthermore, thimerosal increased the maximal response of the residual relaxations to both ACh (from 29.8±8.1% to 42.1±6.1%, P<0.05, n=10, Figure 5C) and A23187 (from 56.9±3.5% to 70.4±3.5%, P<0.05, n=5, Figure 5D).

Concentration-response curves to ACh in the presence of L-NAME (300 µmol/L) and thimerosal (300 µmol/L) showed significant reductions in maximal responses to 18.8±4.9% (n=4) in the presence of Gap 27 peptide (300 µmol/L) although EC50 values remained similar at
0.17±0.05 μmol/L (n=4, Figure 5C). A23187-induced relaxations observed in the presence of L-NAME and thimerosal were not significantly affected by Gap 27 peptide in terms of either the EC₅₀ value (35±17 nmol/L, n=4) or maximal response (66.9±2.1%, n=4, Figure 5).

**Sandwich Preparations**

Relaxations of sandwich preparations to ACh exhibited an EC₅₀ of 1.1±0.27 μmol/L and a maximal relaxation of 21.8±2.7% of phenylephrine-induced tone (n=4, Figure 6A). In marked contrast to the intact ring studies, ACh-induced relaxations were almost abolished in the presence of L-NAME, whereas preincubation with Gap 27 peptide was without effect (Figure 6A). A23187 caused concentration-dependent relaxations of the sandwich preparations, with maximal relaxations constituting 25.4±3.0% of developed tension with an EC₅₀ value of 0.24±0.09 μmol/L (n=8, Figure 6B). In contrast to intact ring studies, preincubation with L-NAME had no effect on A23187-induced relaxations, whereas ONO-RS-082 had no effect on the EC₅₀ value (0.16±0.08 μmol/L, n=4) but markedly attenuated the maximal relaxation to 11±5.8% (P<0.05, n=4, Figure 6B). As in the case of ACh, Gap 27 peptide was without effect on either L-NAME-sensitive or -insensitive relaxations to A23187 in sandwich preparations (Figure 6B).

**Vascular Effects of EETs**

The effects of 3 EET regioisomers, 5,6-, 11,12-, and 14,15-EET, were examined in rabbit superior mesenteric arterial ring preparations. Only 5,6-EET evoked concentration-dependent relaxations of endothelium-intact rings that were abolished following endothelial denudation of the vessel (Figures 7 and 8). Addition of either L-NAME (300 μmol/L) or Gap 27 peptide (300 μmol/L) significantly attenuated these endothelium-dependent responses (P<0.05 [n=4], and P<0.05 [n=6], respectively), and in combination L-NAME...
and Gap 27 peptide abolished 5,6-EET-induced relaxations (n=4, Figures 7 and 8A). Preincubation of ring preparations with either 5,6-, 11,12-, or 14,15-EET (5 μmol/L for each agent) had no effect on either ACh- or A23187-induced relaxations or maximal relaxations (n=4 for each agent, Figure 9).

**Discussion**

We have used isolated ring and sandwich preparations to characterize the role of GJC in the NO-independent responses of the rabbit mesenteric artery to ACh and A23187. The major findings are that EDHF-type relaxations evoked by ACh are attributable to an agent that preferentially crosses between endothelium and smooth muscle via gap junctions, and that a similar agent also mediates relaxation to A23187, but transfer to smooth muscle then involves diffusion via the extracellular space. The formation and/or mode of action of this mediator(s) is intimately related to the metabolism of phospholipids by a Ca²⁺-dependent PLA₂.

Previous studies have shown that heterocellular GJC between the endothelium and smooth muscle contributes to NO-independent relaxations of rabbit conduit arteries. The present experiments confirm that Gap 27 peptide, which contains the SRPTEK motif common to the second extracellular loop of connexins present in the vascular wall, attenuates L-NAME-insensitive relaxations to ACh in rabbit mesenteric artery. In ring preparations, ≈60% of the initial relaxation to ACh was mediated by NO, whereas in sandwich preparations, in which GJC cannot contribute to responses, relaxations were exclusively NO dependent. In marked contrast, only ≈15% of the relaxation evoked by A23187 was mediated by NO, whereas in sandwich preparations, relaxation was not susceptible to inhibition by L-NAME. Furthermore, Gap 27 peptide was completely without effect on the responses to A23187 either in rings or in sandwich preparations. These findings indicate that, unlike ACh, A23187 mediates relaxation predominantly through the release of a freely diffusible factor, which is not NO, into the extracellular space. This conclusion is supported by comparison of the time course of the relaxations to the 2 agents. At concentrations producing equivalent mechanical responses in the presence of L-NAME, relaxations to A23187 were ap-
proximately twice as slow as those to ACh. This is consistent with the idea that the EDHF released by A23187 would have to negotiate 2 cell membranes before exerting a relaxant effect on vascular smooth muscle.

Our findings are consistent with observations by Plane and coworkers, who found that A23187, but not ACh, evokes the release of a diffusible, L-NAME–insensitive factor from the endothelium in sandwich preparations of rabbit femoral artery. These authors suggested that the EDHF released from this artery type was stimulus specific. In the present study, however, NO-independent relaxations to ACh and A23187 were both abolished by the structurally unrelated Ca\(^{2+}\)-dependent PLA\(_2\) inhibitors ONO-RS-082 and AACOCF\(_3\) but not by the Ca\(^{2+}\)-independent PLA\(_2\) inhibitor, HELSS. Although the experimental protocols cannot differentiate between activation of PLA\(_2\) within the endothelium or smooth muscle cell, the simplest explanation of these findings is that a closely similar agent mediates EDHF-type responses to ACh and A23187. Alternatively, if different mediators are involved, the production and/or action of both are crucially dependent on activation of PLA\(_2\) by Ca\(^{2+}\) ions. Inhibition of the Ca\(^{2+}\)-dependent cytosolic form of PLA\(_2\) has also previously been shown to attenuate L-NAME–insensitive relaxations to ACh, histamine, and bradykinin in the perfused rat coronary and mesenteric beds. Cohen and colleagues have suggested that residual NO synthesis in the presence of the NO synthase inhibitors L-NAME and L-\(N^\circ\)-nitroarginine could account for endothelium-dependent hyperpolarization. We excluded this possibility, however, by demonstrating that the selective inhibitor of guanylate cyclase, ODQ, did not modulate the relaxations to ACh and A23187 observed in the presence of L-NAME. The ability of ONO-RS-082 and AACOCF\(_3\) to virtually abolish L-NAME–insensitive relaxations, without affecting NO-mediated relaxations, provides further evidence that L-NAME completely inhibits NO formation in the rabbit mesenteric artery at the concentration used.

Several possibilities may be advanced to explain why A23187-induced relaxations were not susceptible to inhibition by Gap 27 peptide. First, it is possible that incorporation of A23187 into the plasmalemma alters local membrane structure and phospholipid metabolism, allowing easier access of an EDHF linked to the activity of a Ca\(^{2+}\)-dependent PLA\(_2\) to the extracellular space. Second, there is evidence that molecules of A23187 aggregate in lipid bilayers to form channels, as well as behaving as a carrier ionophore with selectivity for Ca\(^{2+}\) ions, that could potentially allow egress of an EDHF into the extracellular space and its subsequent diffusion to adjacent smooth muscle, thereby “bypassing” gap junctions. Third, A23187 has been shown to cause closure of gap junctions between endothelial cells, a mechanism that could promote the egress of an EDHF via the membrane.

Figure 5. Concentration-response curves showing the effects of thimerosal and Gap 27 peptide on ACh- and A23187-induced relaxations in endothelium-intact rings. A and B, Thimerosal (300 nmol/L) significantly increased the potency of ACh (n=5) and A23187 (n=4) but did not affect maximal relaxations. Gap 27 peptide (300 μmol/L) did not reverse the thimerosal-induced increase in potency to ACh but significantly reduced the maximal relaxation. C and D, Thimerosal significantly enhanced L-NAME–insensitive relaxations to both ACh (n=10) and A23187 (n=6). Gap 27 peptide significantly reduced the thimerosal-enhanced relaxations to ACh but not to A23187 (n=4 for both agents).
other cell types, ACh has also been reported to promote closure of gap junctions, but clearly this cannot be an important factor in our experiments, as Gap 27 peptide was highly effective in reducing relaxations to ACh. Conventionally, increases in \([\text{Ca}^{2+}]_i\) have been thought to mediate closure of gap junctions. However, a G-protein/tyrosine kinase-dependent mechanism linked to activation of receptors has recently been shown to promote the closing of gap junctions in cells expressing connexin 43, independently of elevations in local \([\text{Ca}^{2+}]_i\). Conversely, other evidence suggests that elevated \([\text{Ca}^{2+}]_i\) may also open gap junctions. In cardiac myocytes, which express connexin 43 as the dominant connexin protein, channel permeability increases monotonically as a function of \([\text{Ca}^{2+}]_i\), over the physiological range of 0.1 to 1 \(\mu\text{mol/L}\), and decreases only for \([\text{Ca}^{2+}]_i\) within the supraphysiological range. Further experiments are therefore necessary to elucidate the principal mechanisms gating heterocellular GJC between endothelial and vascular smooth muscle cells.

Cascade bioassay experiments have demonstrated that NO inhibits the formation and/or release of EDHF by endothelial cells. This inverse relationship between the activities of the 2 mediators may explain why ONO-RS-082 and AACOCF3 had no effect on ACh-evoked relaxations in ring preparations in the absence of L-NAME but reduced the potency of A23187. Under these conditions, ACh-induced responses were substantially more dependent on NO synthesis than those evoked by A23187, thus suggesting that NO masks the effects of PLA2 inhibition by suppressing EDHF activity. In the presence of L-NAME, however, EDHF activity will be enhanced, and relaxations to ACh then become susceptible to ONO-RS-082 and AACOCF3, as observed experimentally. The positive correlation between the magnitude of the ACh-
induced relaxations obtained in the presence and absence of Gap 27 peptide is also consistent with a dynamic interaction between NO and EDHF, with inhibition of GJC having a smaller effect in the presence of high levels of NO release. A new observation was that in preparations exhibiting large relaxations to ACh in the absence of L-NAME, the subsequent L-NAME–insensitive response to this agonist was small. This suggests that EDHF-type relaxations are chronically diminished in preparations exhibiting high NO activity, although the mechanisms underlying this downregulation remain to be determined.

Activation of PLC following agonist stimulation results in generation of inositol trisphosphate (IP$_3$) and release of Ca$^{2+}$ from internal stores. The resulting increase in [Ca$^{2+}$], activates the Ca$^{2+}$-dependent cytosolic form of PLA$_2$, resulting in cleavage of arachidonic acid from membrane glycerophospholipids. Activity of PLC also generates diacylglycerol (DAG), which can be hydrolyzed by a DAG lipase to provide a second source of arachidonic acid. However, inhibition of NO-independent relaxations to ACh by U-73122 is unlikely to reflect loss of DAG as a substrate for arachidonic acid production, as the DAG lipase inhibitor, RHC-80267, has no effect on EDHF-type responses in the perfused rat heart and mesenteric prearteriolar bed.

Further evidence for the common identity of the EDHF released by ACh and A23187 was obtained with the sulfhydryl agent thimerosal, which has previously been reported to hyperpolarize porcine coronary arterial smooth muscle in an endothelium-dependent manner and enhance the release of an EDHF from canine carotid artery in cascade bioassay. In the present study, low concentrations of thimerosal enhanced relaxations to ACh and A23187 both in the absence and presence of L-NAME and indomethacin. Importantly, the potentiation of EDHF-type relaxations to ACh in the presence of L-NAME remained susceptible to inhibition by Gap 27 peptide, whereas those to A23187 were not. The effects of thimerosal can consequently be attributed to factors other than alterations in the pathways allowing transfer of EDHF between the endothelium and smooth muscle by ACh and through second-messenger–independent mechanisms. Activation of PLC also generates diacylglycerol (DAG), which can be hydrolyzed by a DAG lipase to provide a second source of arachidonic acid.

Figure 8. Effects of 3 regioisomeric EETs, 5,6-EET, 11,12-EET, and 14,15-EET, on vascular tone in endothelium-intact rings of rabbit mesenteric artery. A, 5,6-EET (100 nmol/L to 3 μmol/L) evoked concentration-dependent relaxations (n=6) that were almost abolished following endothelial denudation (n=5). L-NAME (n=4) and Gap 27 peptide (n=6) alone significantly attenuated 5,6-EET-induced relaxations (P<0.05 for both), and in combination they reduced relaxation to the level seen in endothelium-denuded preparations (n=4). B, Neither 11,12-EET nor 14,15-EET induced relaxations of endothelium-intact or -denuded rings.
A23187. Two known mechanisms of action of thimerosal could contribute to an apparent increase in EDHF activity. First, thimerosal inhibits the acyl-coenzyme A:lysolecinin acyltransferase, thereby elevating free levels of arachidonic acid within the cell, an action that would be consistent with the inhibition of relaxation observed with ONO-RS-082 and AACOCF3. Second, nanomolar concentrations of thimerosal equivalent to those used in the present study (5 μmol/L, n=4 for all 3 agents) on relaxations to either ACh (A) or A23187 (B).

Figure 9. Concentration-response curves showing the effect of EETs on relaxations to ACh and A23187 in endothelium-intact rings. There was no effect of a 30-minute preincubation with any of the EETs studied (5 μmol/L, n=4 for all 3 agents) on relaxations to either ACh (A) or A23187 (B).

unknown, effects such as sensitization of vascular smooth muscle to the action of EDHF, but these would not detract from the logic of our argument.

Several groups have suggested that EDHF could be an EET metabolite of arachidonic acid, synthesized by an endothelial cytochrome P-450 epoxygenase, that evokes smooth muscle relaxation through activation of K+ channels and subsequent membrane hyperpolarization. Other workers, however, have reported that relaxation is observed only with micromolar concentrations of EETs, and EETs do not induce hyperpolarization and relaxation in all artery types. A number of groups have demonstrated that inhibitors of cytochrome P-450 attenuate EDHF-mediated relaxations, consistent with the involvement of an EET, but it has become apparent that such inhibitors can also directly inhibit K+ channel activity. Furthermore, highly specific cytochrome P-450 inhibitors appear to have little effect on NO-independent relaxations in rat mesenteric arteries. In the present study with rabbit arteries, micromolar concentrations of 11,12-EET and 14,15-EET were found to be devoid of vasorelaxant activity, and relaxation to 5,6-EET was a strictly endothelium-dependent phenomenon. Other studies have also shown significantly more potent dilator effects of 5,6-EET than 14,15-EET in rat intestinal and cat cerebral microvessels. 5,6-EET may promote Ca2+ influx and stimulate NO release from endothelial cells, a mechanism that seems to operate in rabbit mesenteric arteries, as its effects were partially susceptible to inhibition by L-NAME. A surprising new finding, however, was that L-NAME–insensitive relaxations induced by 5,6-EET were abolished by Gap 27 peptide. In a recent study, Weintraub et al reported that preincubation with EETs potentiates endothelium-dependent relaxations to bradykinin in porcine coronary arteries. In the present study we repeated this experiment and failed to demonstrate potentiation with 5,6-, 11,12-, or 14,15-EET after preincubation for 30 minutes at a concentration of 5 μmol/L. Taken together, our findings therefore provide no evidence that the biologically active EDHF in rabbit arteries is an EET.

We conclude that ACh and A23187 both stimulate the production of an EDHF in rabbit superior mesenteric artery the vasodilator activity of which crucially depends on GIC in the case of ACh and on extracellular diffusion in the case of A23187. The similarity of the effects of 2 structurally dissimilar Ca2+-dependent inhibitors of PLA2 and thimerosal on relaxations obtained to ACh and A23187 point to the operation of common mechanistic pathways. The primary signaling mechanism for relaxation appears to be chemical rather than electrical in nature. The exact identity of the EDHF(s) has yet to be established, and it remains to be determined at which point distal to activation of PLA2 an active agent is mobilized and within which cell type. Since the central pore of gap junctions is aqueous and thought to allow preferential diffusion of small, water-soluble charged molecules, a role for hydrophobic arachidonic acid derivatives, such as EETs, might be challenged on a purely physiochemical basis.

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Iain R. Hutcheson, Andrew T. Chaytor, W. Howard Evans and Tudor M. Griffith

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